EXHIBIT A

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IL-1β decreases the elastic modulus of human tenocytes

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[1]*Flexcell International Corp., Hillsborough;* [2]*Joint Department of Biomedical Engineering, University of North Carolina, Chapel Hill, and North Carolina State University, Raleigh;* [3]*Orthopaedic Research Laboratories, Departments of Surgery and Biomedical Engineering, Duke University Medical Center, Durham; and Departments of* [4]*Orthopaedics and* [5]*Applied and Materials Sciences, University of North Carolina, Chapel Hill, North Carolina*

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Qi, Jie, Ann Marie Fox, Leonidas G. Alexopoulos, Liqun Chi, Donald Bynum, Farshid Guilak, and Albert J. Banes. IL-1β decreases the elastic modulus of human tenocytes. *J Appl Physiol* 101: 189–195, 2006. First published April 20, 2006; doi:10.1152/japplphysiol.01128.2005.—Cellular responses to mechanical stimuli are regulated by interactions with the extracellular matrix, which, in turn, are strongly influenced by the degree of cell stiffness (Young's modulus). It was hypothesized that a more elastic cell could better withstand the rigors of remodeling and mechanical loading. It was further hypothesized that interleukin-1β (IL-1β) would modulate intracellular cytoskeleton polymerization and regulate cell stiffness. The purpose of this study was to investigate the utility of IL-1β to alter the Young's modulus of human tenocytes. Young's modulus is the ratio of the stress to the strain, E = stress/strain = $(F/A)/(\Delta L/L_0)$, where L_0 is the equilibrium length, ΔL is the length change under the applied stress, F is the force applied, and A is the area over which the force is applied. Human tenocytes were incubated with 100 pM recombinant human IL-1β for 5 days. The Young's modulus was reduced by 27–63%. Actin filaments were disrupted in >75% of IL-1β-treated cells, resulting in a stellate shape. In contrast, immunostaining of α-tubulin showed increased intensity in IL-1β-treated tenocytes. Human tenocytes in IL-1β-treated bioartificial tendons were more tolerant to mechanical loading than were untreated counterparts. These results indicate that IL-1β reduced the Young's modulus of human tenocytes by disrupting the cytoskeleton and/or downregulating the expression of actin and upregulating the expression of tubulins. The reduction in cell modulus may help cells to survive excessive mechanical loading that may occur in damaged or healing tendons.

interleukin-1β; cell modulus; tendon; actin; tubulin

CELLS IN NATURAL ENVIRONMENTS are subjected to a complex biomechanical environment, including tension, compression, and fluid shear stress. Evidence indicates that mechanical signals play critical roles in cell differentiation, proliferation, tissue development, skeletal maintenance, and recovery postsurgery (7, 8, 44). Mechanical signals are transduced in cells and regulate their responses to chemical stimulation (3, 7, 14). The mechanisms involved in this cross talk between mechanical and chemical signals are still poorly understood (7, 37).

In part, cells transduce mechanical stimuli through deformation of the cytoskeleton (20–24). The deformability of a cell is determined by a number of factors, including residual tensile "prestress" (residual stress) in its cytoskeleton, which is influenced by the stiffness of the matrix, attachment of the cell to the matrix, cell-cell connections, and contractility, which together determine the elastic stiffness [Young's modulus, the ratio of the stress to the strain, E = stress/strain = $(F/A)/(\Delta L/L_0)$, where L_0 is the equilibrium length, ΔL is the length change under the applied stress, F is the force applied, and A is the area over which the force is applied] of the cell (15, 27). A body of evidence is growing, supporting the idea that the tensile prestress in the cytoskeleton affects a cell's response to mechanical stimulation and therefore to chemical signals (44). Therefore, modulation of cytoskeletal stress may play an important role in tissue development, recovery postsurgery, and in the manufacture of engineered tissues in vitro. Agents, such as interleukin (IL)-1β, that can modulate the cell's cytoskeletal prestress state will be important in regulating the phenotype and thus the fabrication of engineered tissues.

IL-1β treatment increases the secretion and expression of metalloproteinases (MMPs)-1, -2, -3, -9, and -13 in tenocytes (3, 10, 37), bone cells (30), and chondrocytes (26). Most of these studies were focused on the degradation of matrix induced by IL-1β. No studies addressed the effects of IL-1β on the Young's modulus of cells, although recent studies show that IL-1α increases the F-actin content of articular chondrocytes (32). Since IL-1β accelerates the degeneration of matrix, it should also reduce matrix stiffness and cell-matrix attachment. The Young's modulus of cells may also be altered by IL-1β in response to changes in matrix properties or cell attachment. Cell modulus is believed to be mainly determined by the distribution and structure of the cytoskeleton, which is composed of microfilaments, intermediate filaments, and microtubules (33, 35). Disruption of the cytoskeleton dramatically changed the cell modulus (33, 35, 43). Therefore, we hypothesized that IL-1β treatment would reduce the cell modulus by altering the structure of the cytoskeleton and/or regulating the expression of cytoskeletal proteins. A corollary of this hypothesis was that a cell would increase its elasticity (reduce stiffness), facilitate stretching, and better tolerate mechanical loading. In this study, the effects of IL-1β on the Young's modulus of human tendon internal fibroblasts (HTIFs) and cell viability under extreme mechanical conditions were investigated.

MATERIALS AND METHODS

Cell culture. HTIFs were isolated after surgery from discarded human tendon tissues, as described previously (5). HTIFs from *passages 2* to *4* from three different patients were used in this study (*patient 1*, 2-yr-old male, flexor digitorum superficialis tendon; *patient 2*, 84-yr-old female, Dupuytren's contracture; *patient 3*, 77-yr-old

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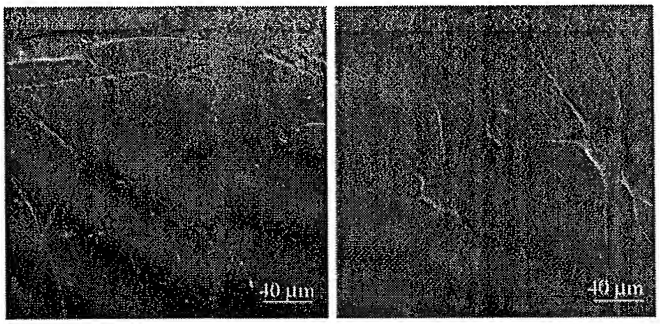

Fig. 1. Differential interference contrast (DIC) images of human tendon internal fibroblasts (HTIFs) treated with or without interleukin-1β (IL-1β). *Left*: control HTIFs; *right*: HTIFs treated with 100 pM IL-1β for 5 days. The cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS). The DIC images were recorded using a LeicaSP2 AOBS confocal microscope. IL-1β treatment changed cell shape dramatically; most of the cells were changed to a stellate shape with multiple processes extending from the central body of the cells. Scale bar is 40 μm.

female, flexor carpi radialis tendon). HTIFs were maintained in medium 199 (GIBCO, Grand Island, NY) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 20 mM HEPES (pH 7.2, GIBCO), and 1% penicillin/streptomycin solution (GIBCO). HTIFs were allowed to attach and spread for 24 h before addition of 100 pM recombinant human IL-1β. The serum concentration was reduced from 10 to 2%. Culture medium was changed daily. On *day 5* (*day 0* is the day when IL-1β was added), cells were released from the culture dishes with 1% collagenase II (Worthington, Lakewood, NJ) at 37°C for 30 min. The released cells were sedimented and suspended in serum-free medium 199.

Measurement of Young's modulus of HTIFs. The Young's modulus of at least 15 cells from each group was measured using a micropipette aspiration technique (16, 35). With this technique, real-time measurements of pressure and deformation can be made and, in conjunction with theoretical models, can be used to determine the intrinsic mechanical and volumetric properties of a single cell. In brief, the solution and cells were placed in a chamber that allowed for the entry of a micropipette from the side. Micropipettes were made by drawing out glass capillary tubes (A-M Systems, Carlsborg, WA) with a pipette puller (David Kopf Instruments, Tujunga, CA) and fracturing them on a microforge to an inner diameter of ~10 μm. The micropipettes and bottom coverslip of the microscope chamber were coated with Sigmacote (Sigma, St. Louis, MO) to prevent cell adhesion. Pressures were applied to the surface of a tenocyte through the micropipette with a custom-built adjustable water reservoir and measured with an in-line pressure transducer having a resolution of 1 Pa (model no. DP15–28, Validyne Engineering, Northridge, CA), as described previously. During the application of pressure, video images of cell aspiration into the micropipette were recorded on an S-VHS video cassette recorder at 60 fields/s with a charge-coupled device camera (COHU, San Diego, CA), through a bright-field microscope (Diaphot 300, Nikon, Melville, NY), using a ×40 oil immersion objective (numerical aperture 1.25; Nikon). The applied pressures and times were displayed on a video monitor using a digital multiplexer (Vista Electronics, Ramona, CA) and recorded to videotape. The length of cell projection into the micropipette and the micropipette inner diameter were measured with a video caliper system (resolution ±0.2 μm).

Staining microfilaments and microtubules. HTIFs were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS), pH 7.2, at room temperature for 15 min, permeabilized with 0.1% Triton X-100 in PBS at room temperature for 20 min, and rinsed with PBS twice. Actin filaments (microfilaments) were stained at room temperature for 1 h with rhodamine-phalloidin (Molecular Probes, Eugene, OR, at 1:400 dilution in PBS). The cells were then rinsed with PBS twice and mounted on glass slides. Microtubules were stained with anti-α-tubulin and anti-β-tubulin monoclonal antibodies, respectively (Sigma). The permeabilized HTIFs were blocked with PBS containing 5% BSA and 2% goat serum at room temperature for 2 h and washed with PBS twice. Then the cells were incubated with the first antibody (1:1,000 for α-tubulin, 1:200 for β-tubulin, diluted in PBS) at 4°C overnight. After washing with PBS, the cells were incubated with Alexa Fluor 488-conjugated (α-tubulin) or Alexa Fluor 568 (β-tubulin) goat anti-mouse IgG (Molecular Probes; 1:200 diluted in PBS) at room temperature for 2 h. The cells were washed with PBS and mounted on glass slides. Cells were imaged using a LeicaSP2 AOBS laser scanning confocal microscope (Leica Microsystem, Exton, PA) with a ×40 oil immersion objective.

Expression levels of actin and tubulin using quantitative RT-PCR. Cells were collected on *days 1*, *3*, and *5* after addition of IL-1β. Total RNA was isolated using an RNeasy mini kit (QIAGEN, Valencia, CA), according to the manufacturer's protocol. Complementary DNA was synthesized with SuperScriptII (Invitrogen, Carlsbad, CA). The expression levels of actin and tubulin were determined by semiquantitative RT-PCR using 18S rRNA as an internal control (Ambion, Austin, TX). Primers for actin were 5′-GCCATCCTGCGTCTGGACCTGGCT-3′ (forward) and 5′-GTGATGACCTGGCCGTCAGGCAGC-3′ (reverse) (19). PCR conditions for actin were as follows: 25 cycles at 94°C for 30 s, 60°C for 60 s, and 72°C for 30 s. The size of the PCR product was 227 bp (19). Primers for α_1-tubulin were 5′-CCATCAAGACCAAGCGCAGCAT-3′ (forward) and 5′-CTCATAGGAGTCGATGCCCACCT-3′ (reverse); primers for β_2-tubulin were 5′-ACAGGCAGTTACCATGGAGACAGT-3′ (forward) and 5′-CCAGAGAGTGGGTCAGCTGGAA-3′ (reverse). The size of the PCR products for α_1- and β_2-tubulins was 300 bp. The PCR conditions were as follows: 30 cycles of 94°C for 30 s, 65°C for 60 s, and 72°C for 30 s. These two pairs of tubulin primers were designed based on the sequences of BT006731 and BC063610 (Genebank access number) using a web-based program, Genefisher (http://bibiserv.techfak.uni-bielefeld.de/genefisher/). The PCR products were separated on 2% agarose gels, and the pixel intensity of the bands was quantitated in Photoshop. The relative expression levels of target genes were normalized to 18S rRNA.

Fabrication and mechanical loading of three-dimensional bioartificial tendon cultures. The three-dimensional (3D) bioartificial tendon (BAT) cultures were fabricated in Tissue Train culture plates (Flexcell International, Hillsborough, NC), as described before but at lower

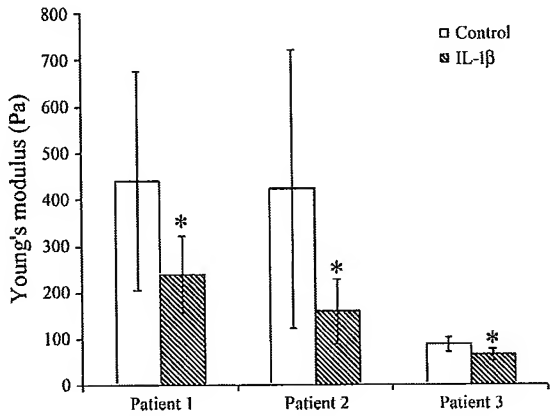

Fig. 2. Young's modulus of at least 15 cells from each group was measured using the micropipette aspiration technique. Human tenocytes from 3 patients were tested. Tenocytes from *patients 1* and *2* showed similar stiffness (modulus); tenocytes from *patient 3* showed a much lower cell modulus. However, the mean Young's modulus of tenocytes from all 3 patients was reduced by IL-1β to 45% (*patient 1*), 63% (*patient 2*), and 27% (*patient 3*) of the control value. Values are means (SD). $*P < 0.05$.

density (50,000 cells/BAT rather than 250,000 cells/BAT) (12). This specialty culture plate allows for the molding of a 3D linear cell-populated matrix gel that is 30 × 4 × 4 mm. In brief, HTIFs were trypsinized, and the cell number was determined using a Coulter particle counter (Beckman Coulter, Hialeah, FL). Cells were mixed with type I collagen (Vitrogen, 2.1 mg/ml; Cohesion, Palo Alto, CA) at 500,000 cells/ml. One hundred microliters (100 μl) of cell-gel suspension were transferred to a space created by vacuum deformation of the rubber membrane into an underlying trough loader jig (30 × 4 × 4 mm space). After gelation, vacuum was released, culture fluid was added, and the cells were cultured in the 3D BAT matrix for 48 h in medium 199 containing 10% FBS. Cells were then brought to quiescence by reducing the serum concentration to 0.5% for 24 h. The cultures were subjected to uniaxial strain for 5 days at 3.5% elongation, 1 Hz, for 1 h per day with Flexcell's Tension Plus cell strain system (model FX4000, Flexcell International, Hillsborough, NC) in the absence or presence of 100 pM IL-1β. After uniaxial loading, the 3D cultures were fixed and stained with rhodamine-phalloidin, as described above. Images were recorded using an Olympus BX60 fluorescence microscope (OPELCO, Dulles, VA).

Determination of nonviable cells. At the termination of the experiments, HTIFs were released from the collagen gels using 1% type II collagenase (Worthington Biochemical, Lakewood, NJ). The number of nonviable cells was determined by a Trypan blue exclusion assay, according to the manufacturer's protocol (Sigma).

Growth curves of human tenocytes. Human tenocytes were plated in 12-well plates and allowed to grow for 24 h in medium 199 (GIBCO) containing 10% FBS (Hyclone, Logan, UT), 20 mM HEPES (pH 7.2, GIBCO), and 1% penicillin/streptomycin solution (GIBCO). Then serum concentration was reduced to 2%, and 100 pM recombinant human IL-1β were added. The media and IL-1β were refreshed daily. Cells were trypsinized, and cell numbers were counted on *days 1*, *3*, and *5*.

Statistics. All experiments were repeated at least three times for each patient. An unpaired Student's *t*-test was used to test

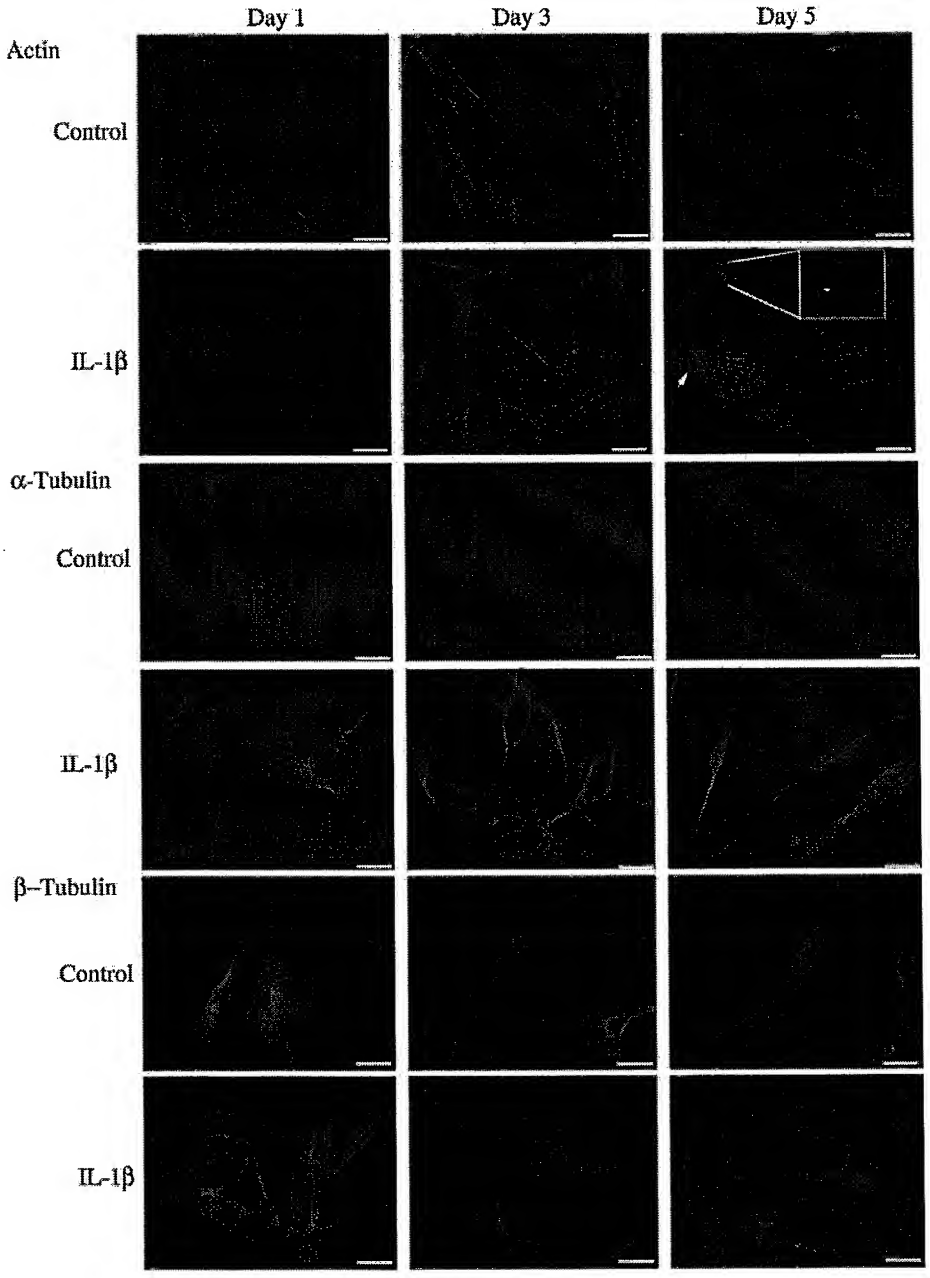

Fig. 3. Effects of IL-1β treatment on the cytoskeleton of HTIFs. The representative results were shown here using the tenocytes from *patient 1*. *Top*: rhodamine-phalloidin staining of actin filaments. On *day 1*, the fluorescence intensity of actin filaments was reduced dramatically by IL-1β. On *days 3* and *5*, numerous short actin filaments were found in IL-1β-treated cells (thin arrow). Also, some thicker, but punctate, actin fibers were formed in ~25% of cells, but were not distributed evenly (thick arrow). *Middle*: immunostaining of α-tubulin. Microtubules were stained with anti-α-tubulin monoclonal antibody and visualized with Alexa Fluor 488-conjugated goat anti-mouse IgG. No obvious change in the structure of microtubules was observed. However, increased staining of microtubules was found from *day 1*. *Bottom*: immunostaining of β-tubulin. Microtubules were stained with anti-β-tubulin monoclonal antibody and visualized with Alexa Fluor 568-conjugated goat anti-mouse IgG. No obvious change in the structure of microtubules or staining intensity was observed. Scale bar is 50 μm.

for significant differences between groups. Changes at a level of $P < 0.05$ were considered significant. The values were expressed as means (SD).

RESULTS

Measurement of Young's modulus. IL-1β treatment changed the cell shape dramatically. Most of the cells changed to a stellate shape with multiple processes extending from the central body (Fig. 1). The mean diameters of collagenase-detached, rounded HTIFs (*patient 1*) from control and IL-1β-treated groups were 17.5 (SD 1.96) and 18.6 μm (SD 2.09), respectively, and were not significantly different ($P = 0.21$, *t*-test). Similar results were found in HTIFs from *patients 2* and *3*. Using this method, previous studies have shown no dependence of the Young's modulus on the cell diameter or on the ratio of cell diameter to micropipette diameter (25). The mean Young's modulus was reduced by IL-1β in HTIFs from all three patients to 45% [*patient 1*, from 439 (SD 235) to 240 Pa (SD 83.0)], 63% [*patient 2*, from 423 (SD 299) to 158 Pa (SD 70.5)], and 27% [*patient 3*, from 87 (SD 15) to 64 Pa (SD 13)] of the control, nontreated levels (Fig. 2).

Effects of IL-1β on the cytoskeleton of HTIFs. Results of rhodamine-phalloidin staining showed that the level of actin filaments in IL-1β-treated cells was dramatically reduced from *day 1* compared with that in control cells (Fig. 3, *top*). Numerous short-actin fibers were found in IL-1β-treated cells from *day 3* (thin arrow in Fig. 3, *top*). On *days 3* and *5*, thicker, but punctate, stress fibers were formed in ~25% of cells, but were not distributed evenly in the cells (thick arrow).

In Fig. 3, *middle* and *bottom*, the structure of microtubules, stained with anti-α-tubulin and anti-β-tubulin monoclonal antibodies, was not changed by IL-1β. However, immunochemical staining for α-tubulin was increased dramatically in IL-1β-treated cells from *day 1*. No obvious changes were found in β-tubulin staining.

Actin and tubulin expression levels in HTIFs with and without IL-1β. Results in Fig. 3 showed that IL-1β changed the levels of actin stress fibers and microtubules. To assess the influence of IL-1β on the steady-state mRNA levels of actin and tubulins, the relative expression levels of actin and tubulins were determined using a semiquantitative RT-PCR method. Results showed that the steady-state mRNA level of actin was reduced by 55% (SD 5.7) on *day 1* but recovered to 90% (SD 4.0) of control on *day 5* (Fig. 4*A*). In contrast to actin, the expression level of α_1-tubulin was upregulated by IL-1β (Fig. 4*B*). α_1-Tubulin expression level was increased by 34 (SD 14), 27 (SD 3.8), and 93% (SD 19), respectively, on *days 1*, *3*, and *5*. The steady-state mRNA level of β_2-tubulin was not changed by IL-1β on *days 1* and *3* and was increased on *day 5* (Fig. 4*C*).

IL-1β increased the tolerance of tenocytes to mechanical loading in 3D cultures. In the absence of IL-1β, the mechanical loading regimen used in this study resulted in cell rounding in most of the rhodamine-phalloidin-stained human tenocytes (Fig. 5*A*). Results of Trypan blue exclusion experiments showed that >90% of stretched cells were nonviable (Fig. 5*B*). However, addition of IL-1β maintained cell viability during 5 days of mechanical loading. Cell shape and density in the load plus IL-1β-treated group were similar to that in the untreated control group, as assessed by rhodamine-phalloidin staining of the F-actin cytoskeleton and Trypan blue exclusion assay (Fig. 5).

The proliferation rate of human tenocytes was not increased in the presence of IL-1β. To confirm that the recovery of cell viability under mechanical loading in the presence of IL-1β was not due to IL-1β-induced cell proliferation, the growth

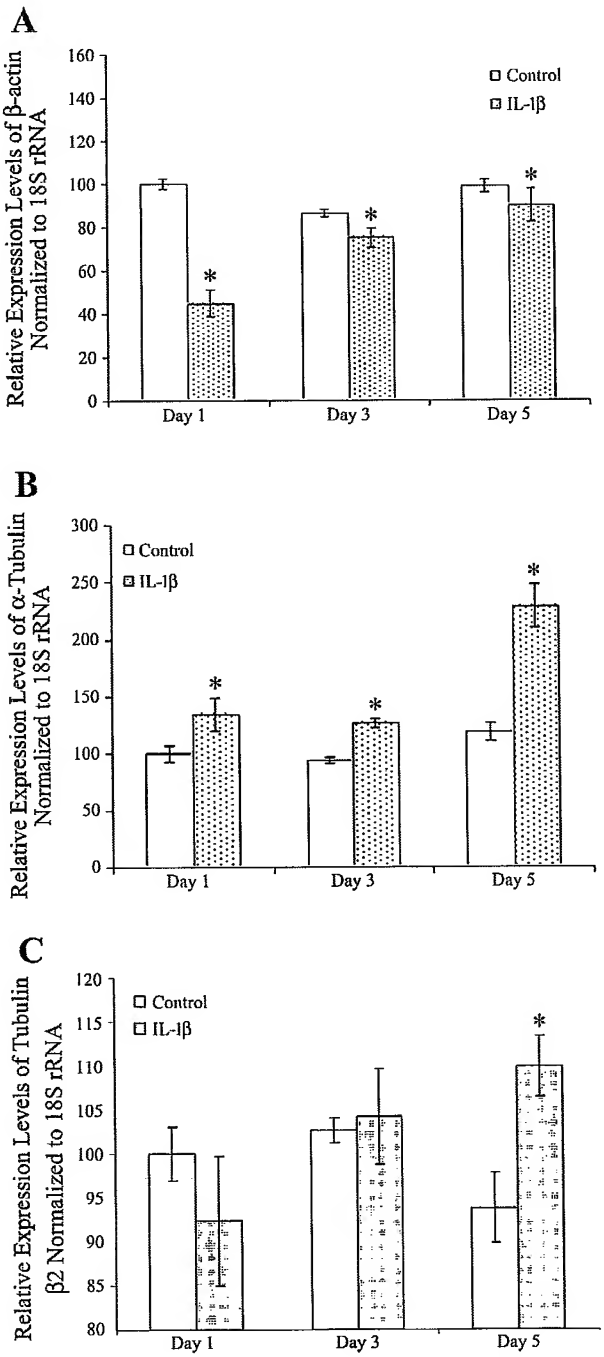

Fig. 4. Relative expression levels of actin and tubulins detected with semiquantitative RT-PCR. Cells were collected at the indicated time points, and semiquantitative RT-PCR was performed. *A*: expression levels of actin. The steady-state mRNA of actin was reduced by 55% (SD 5.7) on *day 1* and recovered to 90% (SD 4.0) of control on *day 5*. *B*: expression levels of α-tubulin. In contrast to actin, the steady-state mRNA of tubulin-α_1 was upregulated by IL-1β by 34 (SD 14), 27 (SD 3.8), and 93% (SD 19), respectively, on *days 1*, *3*, and *5*. *C*: expression levels of β-tubulin. The steady-state mRNA of tubulin-β was not changed by IL-1β on *days 1* and *3* and increased on *day 5*. Values are means (SD). *$P < 0.05$.

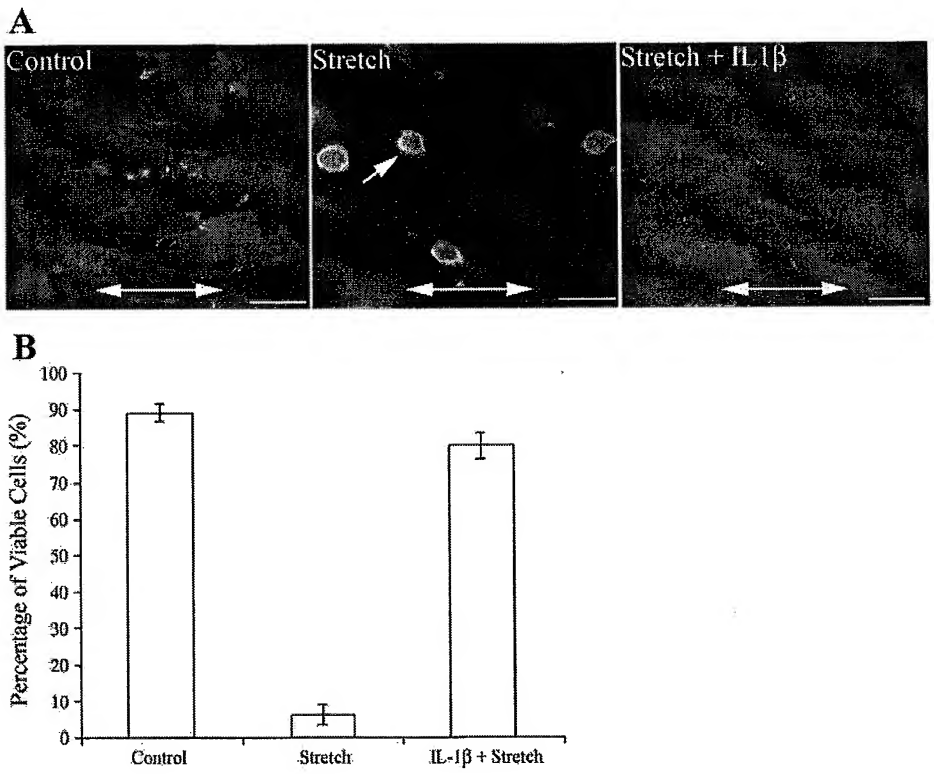

Fig. 5. Effect of stretch on cell viability. *A*: rhodamine-phalloidin staining of three-dimensional (3D) bioartificial tendon cultures of human tenocytes. *Left*: control, nonstretched cells in 3D cultures. *Middle*: cells stretched at 3.5% elongation, 1 Hz, 1 h/day, for 5 days in the absence of IL-1β. The single-headed arrow points to a rounded cell. *Right*: cells stretched with the same conditions as those used for cells in the *middle* panel, but in the presence of 100 pM IL-1β. The direction of applied strain is indicated with two-headed arrows. Scale bar is 50 μm. *B*: Trypan blue exclusion assay. Values are means (SD). Mechanical load reduced the viability of cells grown in 3D collagen gels from 89 (SD 2.4) to 6.4% (SD 2.7); addition of 100 pM IL-1β maintained cell viability [80% (SD 3.4)].

curves of human tenocytes were measured in the absence or presence of IL-1β (Fig. 6). To minimize the interference of serum on IL-1β, the concentration of serum was reduced to 2% (cells at <2% serum did not show obvious growth). Under the experimental conditions, cell numbers were not changed during the 5-day culture period in the IL-1β-treated groups. In the control groups, cell numbers were increased by ~40% on *day 5* compared with *day 1*.

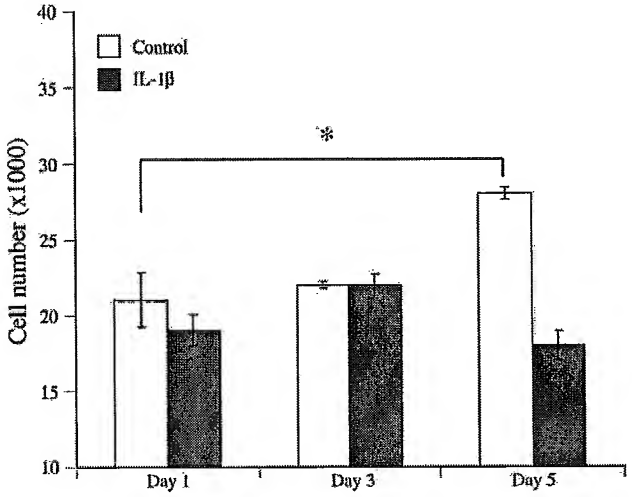

Fig. 6. Growth curves of human tenocytes in the absence or presence of IL-1β. Human tenocytes were plated in 12-well plates at 20,000 per well and allowed to grow for 24 h in complete medium containing 10% serum. Then the serum concentration was reduced to 2%, and IL-1β was added. In the presence of 100 pM of IL-1β, cell number did not show obvious change during the 5-day culturing. In the control groups, cell number was increased 40% on *day 5* compared with *day 1*. Values are means (SD).

DISCUSSION

Mechanical loading plays critical roles in cell differentiation, proliferation, tissue development, skeletal maintenance, and recovery postsurgery (6–8, 12, 44). One way that cells sense mechanical forces is through the deformation of the cytoskeleton (22, 24). In this regard, the mechanical properties of the extracellular matrix relative to those of the cell can significantly affect the micromechanical environment. For example, a mismatch in the Young's modulus of cells relative to the matrix can significantly amplify the cell strains under the same applied load (1, 2, 17). For this reason, it is possible that changes in the mechanical properties of the extracellular matrix (i.e., due to IL-1β stimulation of MMPs) may cause cells to adapt their mechanical properties by reorganizing the F-actin cytoskeleton to maintain a strain setpoint (2). In certain cells, it is believed that mechanical signal transduction is initiated by cell deformation (13, 22, 24). A certain level of tensile prestress will develop upon cell attachment to the matrix, which is determined by the stiffness of the matrix, connections between the cell and matrix (focal adhesion sites), cell-cell connections, and the stiffness (modulus) of cells (15, 27). Changes in any of these properties could alter the intrinsic tension in the cytoskeleton and therefore affect cell responses to mechanical loads.

It has been reported that IL-1β stimulates the expression of MMPs and reduces the stiffness of extracellular matrix due to the degradation of matrix macromolecules (3, 7, 30, 37). It is likely that the cell modulus may be reduced by IL-1β to match the environmental change. In this study, it was shown that IL-1β reduced the Young's modulus for each patient's cells, although there was a large variation in values among patients (~430 vs. ~90.0 Pa). This variation may be due to the differences in individual patients in age, gender, or disease but

may also be due to intrinsic differences among tendons powered by different muscles (25, 34). Cells with a lower Young's modulus are more elastic and therefore may be able to withstand larger deformations before damage or failure. This hypothesis is supported by the results of mechanical loading experiments of 3D cultures in the present study (Fig. 5). The recovery of cell viability was not due to increased cell proliferation rate (Fig. 6). Results of previous studies have shown that mechanical loads stimulated the expression and release of IL-1β in human tenocytes (36). Therefore, the secretion of IL-1β under mechanical loading may represent a mechanism that allows cells to alter their interactions with extracellular matrix under extreme environments by reduction of their modulus and induction of MMPs, which can then be activated to degrade matrix and release cell-matrix contacts (40).

The determinants of mechanical properties of cells are not fully understood; however, it is believed that the elastic modulus is mainly determined by the distribution and structure of the cytoskeleton (35). By using cytoskeleton-disrupting chemicals, it has been found that disrupting F-actin filaments by cytochalasin D decreased the cell modulus (35), whereas disrupting the microtubular network by nocodazol or colcemid increased the cell modulus (43). Similar results were also reported by Kolodney and Wysolmerski (29) and Brown et al. (9). It has also been reported that changing the ratio of α-tubulin to β-tubulin will affect the structure of microtubules (42). The normal ratio of α-tubulin to β-tubulin is 1; overexpression of β-tubulin will disassemble microtubules and induce apoptosis. However, increasing α-tubulin does not obviously change the structure of microtubules (42). These results are consistent with the findings in the present study: that IL-1β may reduce the cell modulus by decreasing/disrupting actin filaments and increasing microtubules. Increasing the ratio of α-tubulin over β-tubulin did not change the structure of microtubules in human tendon cells. In other studies, however, opposite results were reported on the effects of microtubules on cell modulus (33, 35). In these two studies, cytochalasin D (2–3 h postaddition), but not colchicines, reduced the cell modulus of chondrocytes and normal rat kidney fibroblasts. It was also reported that cytochalasin D did not change the elastic modulus of *Strongylocentrotus purpuratus* embryos (<60 min treatment), even though F-actin was severely disrupted (11). These results indicate that the effects of cytoskeleton-disrupting drugs on elastic modulus changes are time sensitive. A more detailed investigation of the time-dependent changes of cytoskeletal structure will be needed to understand the long-term relationship to the elastic modulus of the cell. Also, cytoskeleton-disrupting drugs may initiate signaling pathways in addition to simply disrupting the cytoskeleton (28). However, reducing the cell modulus to a more compliant rather than a stiffer phenotype likely spares a cell from damage due to excessive strain.

Currently, several cell survival pathways have been reported (28, 39, 41). However, the mechanism by which cells survive extreme mechanical loading conditions has not been addressed. IL-1β, as a potential cell survival factor, has been reported to protect blast cells from apoptosis in suspension culture (38). NF-κB pathways activated by proinflammatory factors, such as tumor necrosis factor and IL-1β, also play an important role in anti-apoptosis (31, 41). Therefore, we hypothesize that IL-1β may mediate cell survival under extreme mechanical conditions by activating NF-κB pathway(s), which, in turn, affect the reorganization of the cytoskeleton and reduce the Young's modulus of the cell (4, 18).

For the first time, we report that the mean Young's modulus of HTIFs is reduced by IL-1β. Modulation of the cell's elastic modulus may be useful in mechanical conditioning of tissue engineered constructs and as a treatment postsurgery to increase cell survival.

ACKNOWLEDGMENTS

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DISCLOSURES

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EXHIBIT B

TISSUE ENGINEERING
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Interleukin-1β Increases Elasticity of Human Bioartificial Tendons

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ABSTRACT

Stiffness is an important mechanical property of connective tissues, especially for tissues subjected to cyclic strain *in vivo*, such as tendons. Therefore, modulation of material properties of native or engineered tissues is an important consideration for tissue repair. Interleukin 1-β (IL-1β) is a cytokine most often associated in connective tissues with induction of matrix metalloproteinases and matrix destruction. However, IL-1β may also be involved in constructive remodeling and confer a cell survival value to tenocytes. In this study, we investigated the effects of IL-1β on the properties of human tenocyte-populated bioartificial tendons (BATs) fabricated in a novel three-dimensional (3D) culture system. IL-1β treatment reduced the ultimate tensile strength and elastic modulus of BATs and increased the maximum strain. IL-1β at low doses (1, 10 pM) upregulated elastin expression and at a high dose (100 pM) downregulated type I collagen expression. Matrix metalloproteinases, which are involved in matrix remodeling, were also upregulated by IL-1β. The increased elasticity prevented BATs from rupture caused by applied strain. The results in this study suggest that IL-1β may act as a defense/survival factor in response to applied mechanical loading. The balance between cell intrinsic strain and external matrix strain is important for maintaining the integrity of tendons.

INTRODUCTION

THE DEGREE OF STIFFNESS OR ELASTICITY is an important material property of connective tissues required to fulfill their physiologic functions. *Stiffness* is the resistance of an elastic body to deflection by an applied force. It is proportional to the slope of the linear portion of the stress–strain curve of a material. The steeper the slope, the stiffer the material. Material properties of tendons change during development, remodeling, and wound healing.[1–4] Even the same tendon may show different stiffnesses at different anatomic locations.[3,5] Therefore, the regulation of tendon stiffness is important for proper function *in vivo*. Mechanical properties are also important in engineered tissues, in which strength is needed to produce a functional construct with a modulus comparable to that of native tissue. Consensus in the field is that engineered tendons match the demands of the *in vivo* mechanical environment and facilitate recovery post-surgery.[4,6,7]

The mechanical properties of a tissue are determined by its extracellular matrix composition and orientation.[8] In tendon, the extracellular matrix is mainly composed of type I collagen (65–80% of dry weight) and elastin (1–2% of dry weight).[9,10] Results of mechanical tests show that increased stiffness of tendon is mainly determined by the expression level of type I collagen, but that decreased stiffness (elasticity) is determined by the expression level of elastin.[11] Therefore, differential regulation of the expression levels of type I collagen and elastin in tendon tissues modulates their stiffness.

The expression of type I collagen and elastin can be regulated by many factors, such as transforming growth

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factor-β (TGF-β),[12–15] interleukin-1β (IL-1β),[16,17] heparin,[18] basic fibroblast growth factor,[19] insulin-like growth factor,[20] okadaic acid,[21] ascorbate,[22,23] and exercise.[24] TGF-β and ascorbate have been broadly used in tissue engineering applications to increase the mechanical strength of engineered tissues. However, tissue engineered tendon constructs often rupture during development from excessive cell-generated strains.[25–27] Therefore, we should consider approaches to regulate or reduce the stiffness of engineered tissues during critical periods in their development.

Previous studies showed that matrix metalloproteinases (MMPs) play important roles in extracellular matrix remodeling, which is a critical component of tissue growth and morphogenesis.[28] It is expected that MMPs play important roles in modulating the mechanical properties of engineered tissues. Expression of MMPs is especially important for the remodeling of long-lived proteins, such as elastin.[29]

IL-1β is a proinflammatory factor often found at a site of tendon injury.[30,31] IL-1β has been reported to regulate the expression of elastin in cultured fibroblasts.[17,21] It has been reported to either upregulate or downregulate elastin expression depending on the cell types used. Upregulation of elastin expression by IL-1β in dermal fibroblasts was independent of ongoing protein synthesis, whereas downregulation of the steady-state mRNA level of elastin by IL-1β in rat lung fibroblasts was blocked by cycloheximide.[17,21] Similar results were reported for the regulation of collagen accumulation by IL-1β in dermal fibroblasts, chondrocytes, or foreskin fibroblasts.[32,33] These results indicate that IL-1β may differentially regulate the expression of elastin and collagen. However, no similar study has been reported for other connective tissues, especially for human tenocytes in three-dimensional (3D) cultures.

We and other groups have shown that IL-1β upregulated the expression of collagenases (MMP-1 and -13) in tendon cells.[34,35] It was reported that stress deprivation upregulated the expression of MMP-1, which contributed to the stress-deprivation–induced decrease in failure strain of tendons.[36] Therefore, overexpression of collagenases may reduce the net deposition of type I collagen in the matrix and result in the reduction of ultimate tensile strength (UTS).

These results indicate that overexpression of IL-1β may stimulate the remodeling of extracellular matrix and function as a survival factor to prevent tendon injury caused by extreme or repetitive mechanical loading (overuse). In the present study, human tenocyte-populated bioartificial tendons (BATs) were used as an *in vitro* model to study the regulation of the mechanical properties of engineered tissues by IL-1β. BATs are linear 3D cultures fabricated in a novel, *in vitro* culture system (Tissue Train®, Flexcell International Corporation, Hillsborough, NC).[27,37] It was hypothesized that IL-1β would differentially regulate the expression of elastin and type I collagen, and upregulate the expression of MMPs in tenocytes resulting in increased elasticity of BATs. It was further hypothesized that increased elasticity might prevent construct failure owing to applied mechanical loading. IL-1β may act as a defense/survival factor in early cell responses to mechanical loading.

MATERIALS AND METHODS

Cell culture

Human tendon internal fibroblasts (HTIFs) were isolated from discarded human tendon tissue specimens after surgery as described previously.[38] Five tendon tissues were used in this study. Specimen 1 was a flexor digitorum superficialis tendon from a 26-year-old male; specimen 2 was an extensor pollucis longus laceration specimen from a 31-year-old male; specimen 3 was a left ring finger Dupuytren contracture specimen from an 84-year-old female; specimen 4 was a superficialis tendon from a 2-year-old boy; and specimen 5 was a right palmaris longus tendon from a 48-year-old male. HTIFs from passages 2–4 were maintained in Medium 199 (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 20 mM HEPES pH 7.2, 1% penicillin/streptomycin solution (Gibco).

Fabrication of human tenocyte-populated bioartificial tendons

The BATs were fabricated in 6-well, 35-mm diameter per well, Tissue Train culture plates (Flexcell International) as described previously.[27,37] This special culture plate allows for the molding of a 3D, linear, cell-populated matrix gel that is 25×4×4 mm. Briefly, HTIFs were trypsinized with 0.01% trypsin in PBS pH 7.4 and the cell number determined with a Coulter particle counter (Beckman Coulter, Hialeah, FL). Cells were mixed with type I collagen (Vitrogen, 2.1 mg/mL; Cohesion, Palo Alto, CA) at 2×10^6 cells/mL and 100 μL of the mixture were cast in a trough mold (Trough loader, Flexcell International) centrally located in a Tissue train culture plate (Flexcell International). The plates were placed in a CO_2 incubator at 37°C during the gelation phase. After gelation, the cells were cultured in the 3D matrix for 48 h in 2 mL/well Medium 199 containing 10% FBS.

Treatment of bioartificial tendons with interleukin-1β

Forty-eight h post-gelation, BATs were cultured in medium 199 with 2% FBS in the absence or presence of 100 pM IL-1β for 5 days. The media were changed daily. The effects of IL-1β were investigated in the presence of cycloheximide (10 μg/mL), cytochalasin D (10 μM), or the peptide GRGDTP (100 μg/mL) to ascertain the results of blocking protein synthesis or releasing tension in cells from the inside (actin) or outside (integrin–matrix), respectively. Chemicals were added in the medium at the indicated final concentrations with or without IL-1β (Sigma, Saint Louis, MO).

Mechanical loading of HTIF-populated bioartificial tendons

On day 5, 3 BATs from each group were subjected to a single 30% strain for 10 s using a model FX4000 Flexercell Strain Unit with arctangle loading posts (Flexcell International).[27,37,39] The images of the BATs were then scanned with a scanner controlled by a laptop (ScanFlex, Flexcell International) and the length of BATs was measured using SigmaScan (SPSS Scientific, Chicago, IL) to depict the displacement length poststrain and the kinetics of return to original length of the BATs.

Measurement of mechanical properties of HTIF-populated bioartificial tendons

On day 5, the mechanical properties of BATs were measured using an EnduraTec ElectroForce 3200 system (EnduraTec Systems Group, Minnetonka, MN). Six BATs from each group were excised from Tissue Train culture plates and clamped in specially constructed fixtures that gripped the nylon anchors at each end of a BAT (Fig. 1). The bottom clamp was connected to the load cell and remained stationary. The top clamp was moved upward at a strain rate of 0.1 mm/second. The grip-to-grip distance between the 2 clamps was 7.5 mm and the excursion distance was 10 mm. Two cameras placed at 90 degrees to each other were used to capture specimen images from which the cross-sectional area of BATs was calculated (see Fig. 1). The elastic modulus (the slope of the stress–strain curve, which is proportional to the stiffness), UTS (the maximum strength that the BATs can sustain), and maximum strain (at the failure point) were determined based on the stress–strain curve data (Fig. 2).

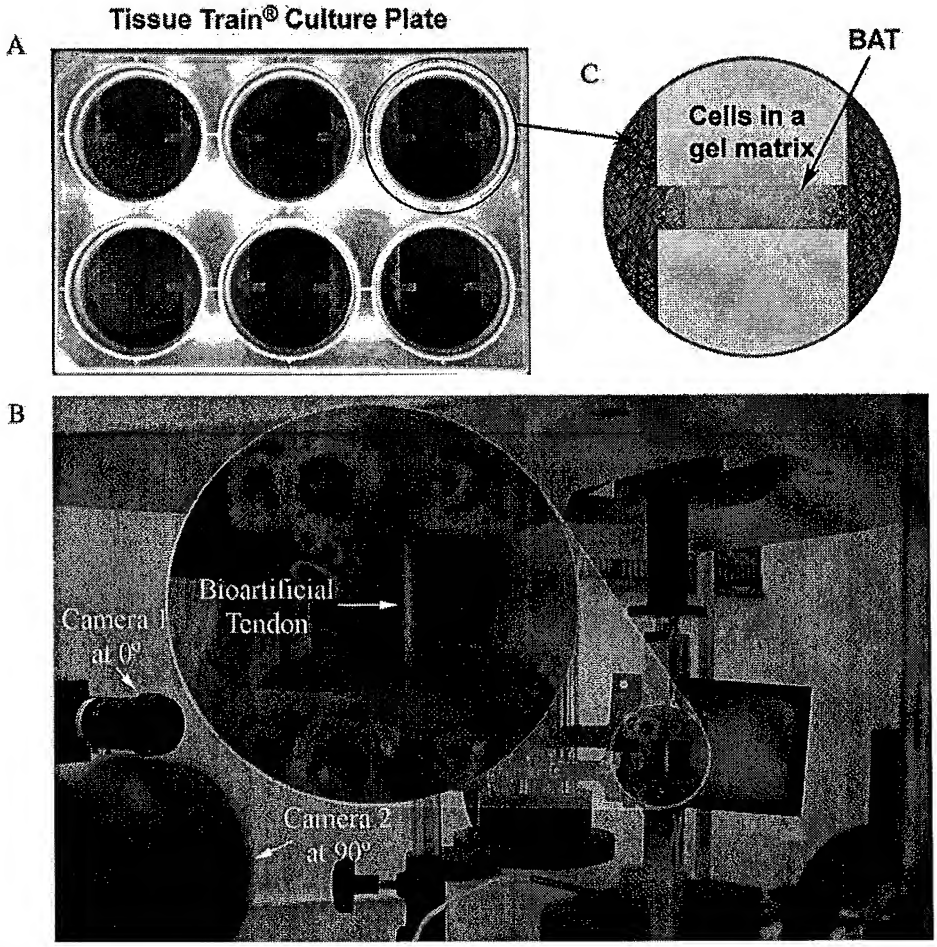

FIG. 1. Mechanical testing of bioartificial tendons (BATs) populated with human tenocytes. An EnduraTEC ElectroForce 3200 system was used to obtain material properties. Two cameras situated at 90 degrees to each other were installed to capture the images of BATs during testing for the calculation of cross-sectional areas. BATs were clamped between 2 fixtures. The bottom clamp was connected to the load cell and was immobile. The top clamp was displaced at a rate of 0.1 mm/second. The distance between the clamp–BAT interfaces (grip to grip) was measured for calculation of strain as $\Delta l/l_0$, where l_0 was the original length of the BAT and Δl was the change in length of the BAT. The UTS and elastic modulus of BATs were calculated based on the stress–strain curves with a correction for specimen cross-sectional area. **(B)** Enlargement of the specimen clamping area. The mechanical properties of 6 BATs from each group were tested and the experiment was repeated 3 times.

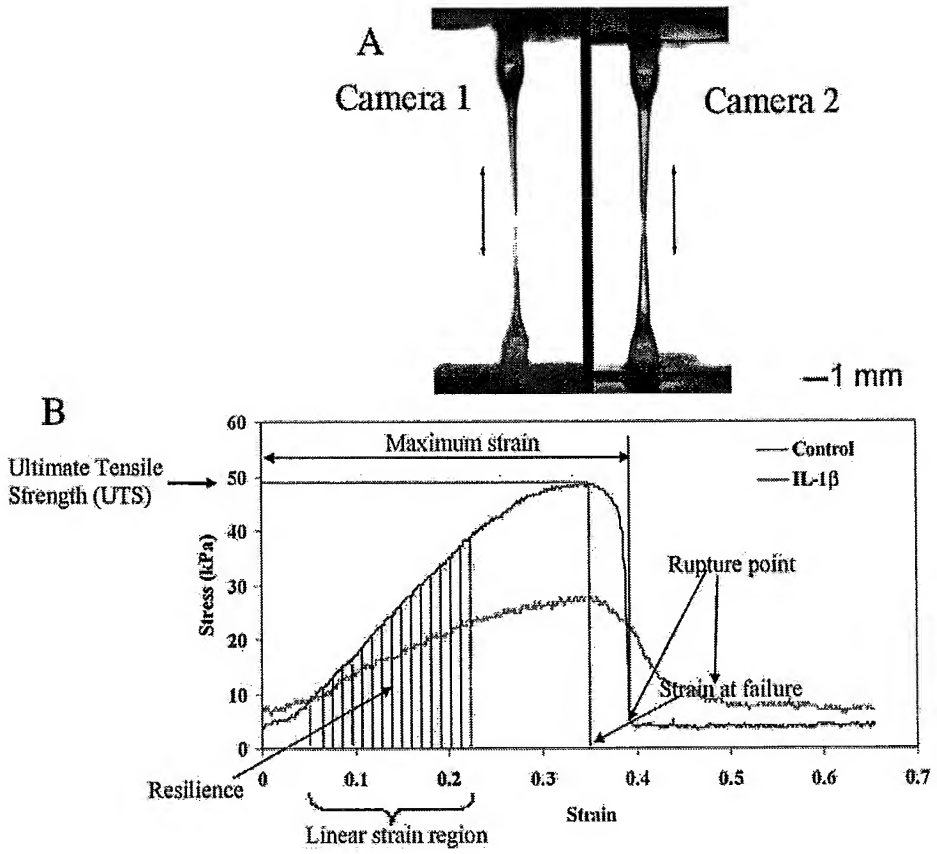

FIG. 2. Stress–strain curve of BATs. (**A**) Image of a BAT during testing. The *arrows* indicate the excursion direction in the images from cameras 1 and 2. (**B**) Data indicate that IL-1β treatment reduced the UTS and elastic modulus of BATs, but increased the maximum strain. The increased maximum strain was due to the increased strain between failure and rupture points. Six BATs from each group were tested and the experiment was repeated at least 3 times. A representative image is shown here. IL: interleukin.

Quantitative reverse transcription PCR

At the indicated time points, 3 BATs from each group were excised from their nylon anchor attachments in Tissue Train culture plates and total RNA was isolated using an RNeasy mini kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. Complementary DNA was synthesized with SuperScriptII (Invitrogen, Carlsbad, CA). The expression levels of elastin and type I collagen were determined by semiquantitative reverse transcriptase (RT)-PCR using 18s ribosomal RNA (rRNA) as an internal control (Ambion, Austin, TX). The multiplex PCR products were separated on 2% agarose gels and the pixel intensity of the bands was quantitated in Photoshop. The relative expression levels of target genes were normalized to that of a 18s rRNA target. The primers used in this study were: elastin forward 5′-TGCTCTTCTCAATCTTGCAGGGTT-3′, reverse 5′-TCCAAGTCCAGGAACAC CAGCA-3′; type I collagen α1 forward 5′-AGACATGTTCAGCTTTGTGG ACCT-3′, reverse 5′-CTTGGTCGGTGGGTGACTCTGA-3′; type I collagen α2 forward 5′-CAAACGGCCTTACT GGTGCCAA-3′, reverse 5′-CAGGAAGACCACGA-GAAC CAGGA-3′. The size of each of the PCR products for these 3 pairs of primers was 300 bp. PCR conditions were as follows: 94°C for 5 min; 25 cycles of 94°C for 30 sec, 65°C for 60 sec, and 72°C for 30 sec; and 72°C for 5 min. The ratio of pair to competimer was 1:9 for elastin and 2:8 for type I collagens.

Immunostaining of elastin and type I collagens in bioartificial tendons

After 5 days of treatment with IL-1β, 3 BATs from each group were fixed with 3.7% formaldehyde at room temperature for 30 min and then permeabilized with 0.1% Triton X-100 at room temperature for 15 min. After washing with phosphate-buffered saline (PBS pH 7.4), BATs were blocked with 5% bovine serum albumin plus 2% goat serum dissolved in PBS at room temperature for 2 h. The primary antibodies (monoclonal antibodies for anti-elastin and type I collagen were purchased from Sigma) were

TABLE 1. BIOMECHANICAL PROPERTIES OF BIOARTIFICIAL TENDONS

	Linear range (%)	*Ultimate tensile strength (kPa)*	*Elastic modulus (kPa)*	*Strain at ultimate tensile strength (%)*	*Maximum strain (%)*
Control	17 ± 3.0	51 ± 3.0	197 ± 52.4	31 ± 4.1	40 ± 2.2
interleukin-1β-treated	18 ± 3.4	34 ± 6.2*	108 ± 49.2*	34 ± 8.7	47 ± 3.5*

*indicates a significance level < 0.05

diluted in PBS at 1: 1000 and incubated with BATs at 4°C overnight. The target proteins were visualized with Alexa Fluor 488 goat anti-mouse IgG (H+L) (for elastin, diluted in PBS at 1:200; Molecular Probes [Eugene, OR]) or Alexa Fluor 568 goat anti-mouse IgG (H+L) (for type I collagen, diluted in PBS at 1:500; Molecular Probes) at room temperature for 2 h. BATs were viewed using a LeicaSP2 AOBS laser scanning confocal microscope (Leica Microsystems, Exton, PA) with a 40× oil immersion objective.

Zymography of matrix metalloproteinases

The conditioned media from each group (3 samples from each group) were collected on day 5 and the gelatinase activity was assayed.[40] Briefly, pig gelatin type A (Sigma, 2 mg/mL) was mixed in a 7.5% SDS-PAGE gel. Twenty microliters of conditioned media were mixed with same volume of 2×SDS sample buffer without dithiothreitol at room temperature for 10 min and separated in the SDS-PAGE gel until the dye-front was near the bottom of the gel. Gelatinases were renatured in 2.5% Triton X-100 dissolved in deionized water at room temperature for 30 min and developed in developing buffer (50 mM Tris-HCl, 0.2 M NaCl, 5 mM $CaCl_2$, 0.02% Brij[35]) at 37°C for 24 h. Gels were stained in 0.5% Coomassie blue R-250 in 50% methanol, 10% acetic acid for 30 min and destained with destaining solution (methanol:acetic acid:dH_2O = 50:10:40). The gels were then scanned and the bands were quantitated in Photoshop.

Statistics

For mechanical testing, 6 BATs from each group were tested and triplicate samples were used in immunostaining and RT-PCR. Each experiment was repeated at least three times. Statistical analyses between test and control groups were performed using SigmaStat software (SPSS Scientific). Significance ($p < 0.05$, indicated as *) was determined by a 1-way repeated ANOVA with Dunnett's *t*-tests. The variances between minus and plus IL-1β under each experimental condition were analyzed using an unpaired Student *t*-test. A *p* value less than 0.05 was deemed significant (indicated as +).

RESULTS

IL-1β treatment increased the elasticity of human tendon internal fibroblast populated bioartificial tendons in 3D collagen gel cultures

The results of biomechanical testing showed that the modulus of BATs was reduced by almost 50% (from 197 ± 52.4 kPa to 108 ± 49.2 kPa; $p < 0.05$) by IL-1β (100 pM) treatment (see Fig. 2). The UTS was reduced by approximately 20% (from 51 ± 8.4 kPa to 34 ± 6.2 kPa; $p < 0.05$; Table 1). The strain at failure of BATs was unchanged by IL-1β (control 31 ± 4.1% versus IL-1β–treated 34 ± 8.7%; $p = 0.59$). However, the total strain (maximum strain) of BATs was extended from 40 ± 2.2% to 47 ± 3.5% (increased 18%; $p < 0.01$) owing to the increased strain between the failure and rupture points (see Fig. 2 and Table 1). The initial linear portion of the stress–strain curve yielded the elastic range of BATs. IL-1β treatment did not change the linear range of BATs (control 17 ± 3.0% versus IL-1β–treated 18 ± 3.4%; $p = 0.61$; see Table 1). Therefore, the resilience (the area under the curve in the linear region in the stress–strain diagram) of BATs was also reduced because of the lower elastic modulus in IL-1β–treated BATs.

Increased elasticity of bioartificial tendons prevented bioartificial tendons from rupture caused by applied strain

All the BATs from the control group were broken by the extreme loading (30% strain for 10 sec), whereas the BATs from the IL-1β–treated group were intact but elongated (Fig. 3A). The elongated BATs recovered to the original length within 10 h (Fig. 3B).

Regulation of the expression of type I collagen and elastin by interleukin-1β

Quantitative RT-PCR showed that the steady-state mRNA levels of both of α1 and α2 chains were downregulated by IL-1β (100 pM) to 37 ± 2.9% (α1; $p < 0.05$) and 31 ± 1.6% (α2;

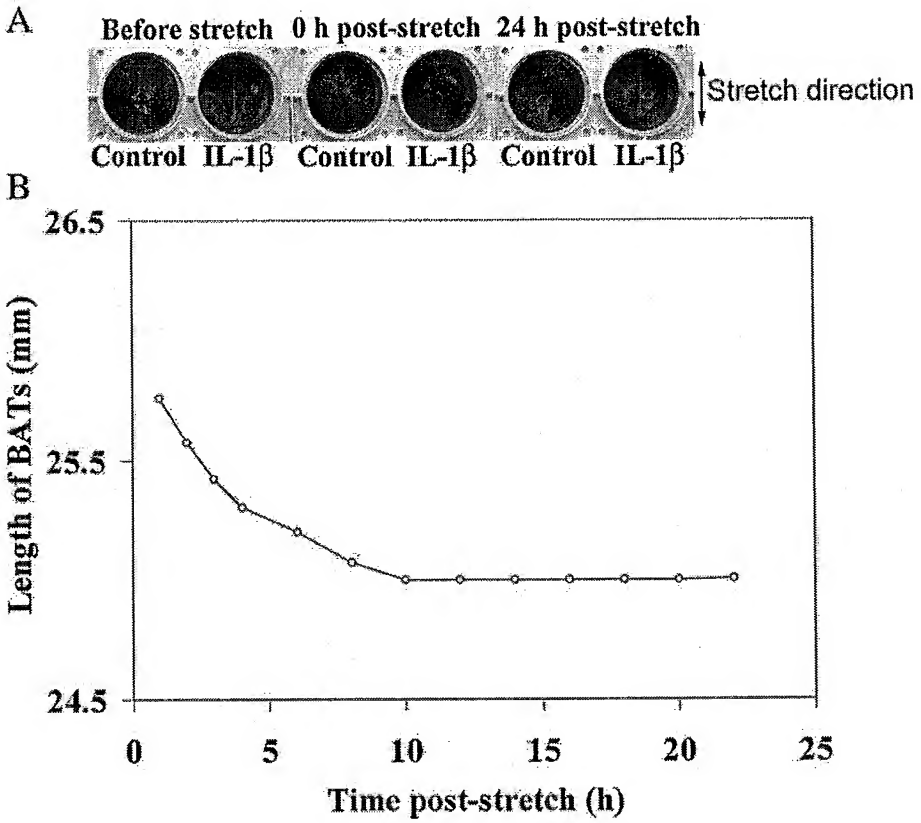

FIG. 3. IL-1β prevented BATs breaking from extreme mechanical loading. Bioartificial tendons (BATs) from control and IL-1β–treated groups on day 5 were subjected to 30% strain and kept at 30% strain for 10 sec. (A) All BATs from control group were broken by 30% strain, whereas the IL-1β–treated BATs were intact but elongated. (B) The recovery curve of elongated BATs showed that the elongated BATs rapidly retracted to the original length within 10 h. This experiment was repeated 3 times. IL: interleukin.

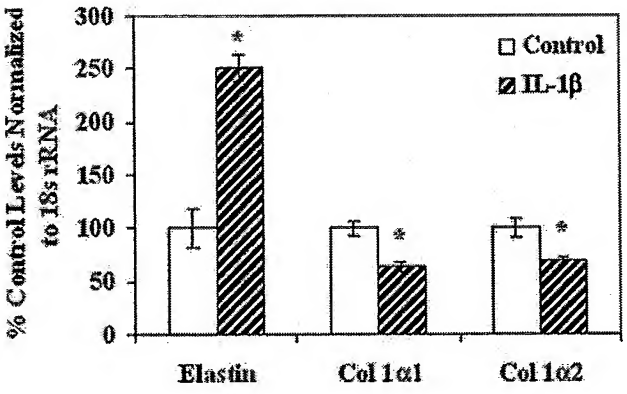

FIG. 4. Quantitative RT-PCR of type I collagen and elastin. Three BATs from each group were excised from the Tissue train culture plates on day 5 post-addition of 100 pM IL-1β. Total RNAs were isolated directly from the BATs. Quantitative RT-PCR was carried out using 18s rRNA as an internal control. The steady-state mRNA of type I collagen chain α_1 was reduced by $36 \pm 2.9\%$ ($p = 0.002$); α_2 chain mRNA was reduced by $31 \pm 1.6\%$ ($p = 0.004$). The steady-state mRNA of elastin was increased by approximately 1.5-fold ($p < 0.001$). The experiment was repeated 3 times. COL: collagen; IL: interleukin.

$p < 0.05$) of control levels, respectively (Fig. 4). In contrast to the downregulation of type I collagen, the steady-state mRNA level of elastin was dramatically increased in IL-1β–treated cells (1.5-fold increase, $151 \pm 7.6\%$; $p < 0.001$; see Fig. 4). The immunostaining results showed that protein levels of type I collagen and elastin were regulated by IL-1β (Fig. 5). The expression levels of type I collagen chains were downregulated to the greatest extent at 24 h post-addition of IL-1β ($72 \pm 6.7\%$ and $69 \pm 2.7\%$ control for α_1 and α_2 chains, respectively). Alternatively, elastin expression was upregulated at 8 h post-addition of IL-1β ($110 \pm 4.3\%$ of control level) and to the greatest extent at 24 h post-addition of IL-1β ($256 \pm 9.4\%$ of control level; Fig. 6). Results of a dose–response experiment showed that type I collagen expression was downregulated by 100 pM IL-1β, but not by 1 or 10 pM IL-1β at 24 h. In contrast, elastin expression was upregulated by IL-1β even at 1 pM (Fig. 7).

Cycloheximide was used to block protein synthesis. Regulation of elastin expression by IL-1β was independent of ongoing protein synthesis (Fig. 8). Cycloheximide alone increased the steady-state level of elastin mRNA.

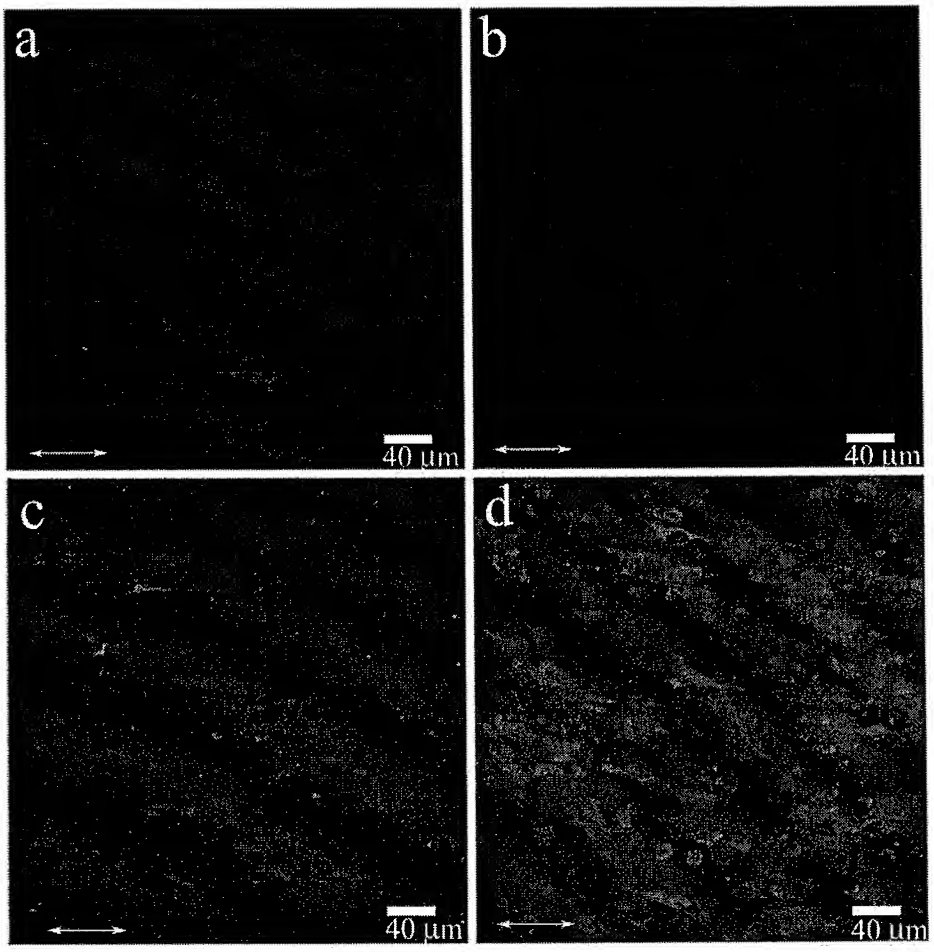

FIG. 5. Immunostaining of type I collagen and elastin in BATs. Three BATs from each group were fixed on day 5 post-addition of 100 pM IL-1β stained with monoclonal anti-human type I collagen or anti-human elastin antibody and visualized with AlexaFluor 488 conjugated (elastin, *green*) or AlexaFluor 568 conjugated (type I collagen, *red*) goat anti-mouse IgG antibody. (**A**) Stained type I collagen in a control BAT. (**B**) Stained type I collagen in an IL-1β–treated BAT. (**C**) Stained elastin in a control BAT. (**D**) Stained elastin in an IL-1β–treated BAT. Scale bar = 40 μm. *Double headed arrow* indicates linear direction of BAT. The experiment was repeated 3 times.

Regulation of the expression of type I collagen and elastin by IL-1β under reduced tension

To determine if the intrinsic tension of tenocytes would affect their response to 100 pM of IL-1β, the peptide GRGDTP and cytochalasin D were used to reduce the intrinsic tension of cells from outside and inside the cells, respectively. The peptide, GRGDTP, is an antagonist of cell attachment to type I collagen, vitronectin, and fibronectin.[41,42] This peptide interferes with integrin-based cell binding to type I collagen. The result is that cells release matrix attachments and reduce their intrinsic tension from the cell exterior (from external integrin–matrix attachments). Cytochalasin D prevents actin polymerization[43] and disrupts the intrinsic tension of cells derived of microfilaments from the cell interior (from internal integrin–cytoskeletal attachments). The results showed that neither GRGDTP nor cytochalasin D blocked the effects of IL-1β on the expression of elastin. Cytochalasin D alone reduced the steady-state mRNA level of elastin (48 ± 2.6% of control; Fig. 9). The steady-state mRNA level of the type I collagen α_1 chain was not affected by either peptide GRGDTP or cytochalasin D, but both peptide GRGDTP and cytochalasin D increased the effect of IL-1β on the expression of α_1 chains. The steady-state mRNA levels of type I collagen α_2 chains were reduced by peptide GRGDTP and cytochalasin D. However, neither cytochalasin D or peptide DRGDTP blocked the effects of IL-1β on the expression of type I collagen α_2 chains.

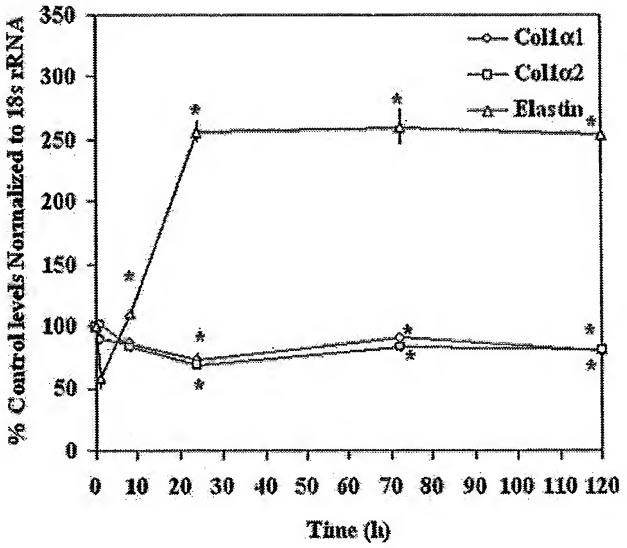

FIG. 6. Time course of IL-1β–induced expression of type I collagen and elastin. The expression of elastin was upregulated from 8 h post-addition of IL-1β and reached a maximum at 24 h. The expression of type I collagen was downregulated by IL-1β from 24 h post-addition of IL-1β. There was no further change on the steady-state mRNA of elastin and type I collagen from 24 h post-addition of IL-1β. $*p < 0.05$. The concentration of IL-1β was 100 pM. Triplicate samples were used at each time point and the experiment was repeated 3 times. COL: collagen.

Matrix metalloproteinase-2 and -9 were upregulated by interleukin-1β

We reported that IL-1β upregulated the expression of MMP-1, -3, and -13 in human tenocytes.[34] In the present study, we investigated the effect of IL-1β on the expression of MMP-2 and -9 in human tenocytes grown in 3D cultures (BATs; Fig. 10). Both MMP-2 and MMP-9 were upregulated by IL-1β. MMP-2 (both latent and active forms) was increased by 2-fold and MMP-9 (both latent and active forms) was increased by 3-fold.

DISCUSSION

A principle of "Functional Tissue Engineering" put forth by Guilak *et al.*[7,44] is that a tissue fabricated for use in the body should match the mechanical environment in which it will function. We agree with this basic principle. However, fabrication processes that involve cell compaction of a provisional gel matrix often result in rupture of the construct by cell-generated forces.[25–27] Therefore, regulating the compaction process by reducing the number of cell matrix attachments or by changing the cell modulus may be a viable strategy for generating a strong but compliant connective tissue. The results in the present study indicate that IL-1β may act as a matrix/cell modulus modifier and increase cell survival in engineered tissues.

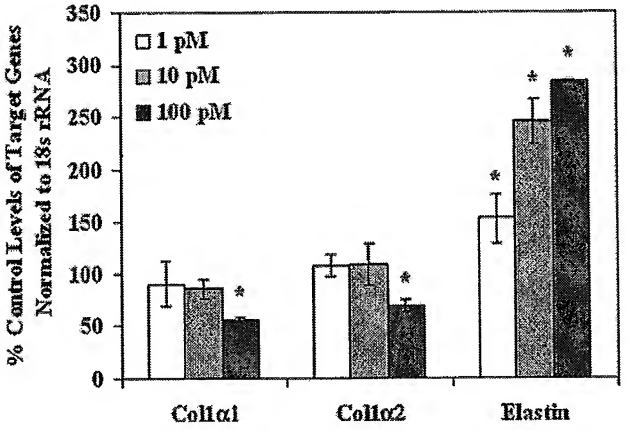

FIG. 7. Dose–response of IL-1β on the regulation of the expression of type I collagen and elastin. BATs were incubated for 24 h with 1, 10, or 100 pM IL-1β and the total RNA was isolated and quantitative RT-PCR was carried out using 18s rRNA as an internal control. Elastin responded to IL-1β at as low as 1 pM concentration, whereas type I collagen responded at much higher concentration (100 pM). $*p < 0.05$. Triplicate samples were used in each data point and the experiment was repeated 3 times. COL: collagen.

It has been reported that IL-1β decreased the steady-state mRNA level of elastin in rat myofibroblasts[45] and lung fibroblasts.[16] This effect was blocked by cycloheximide, an inhibitor of protein synthesis.[16] Mauviel *et al.*[17] reported that IL-1β upregulated elastin gene expression, which was independent of ongoing protein synthesis in dermal fibroblasts.[17] These results indicate that different mechanisms may be involved in the regulation of elastin expression by IL-1β in different cell types. The results of cycloheximide experiments indicate that the same pathway may be activated in the regulation of elastin expression by IL-1β in dermal fibroblasts and tenocytes. The increase in the

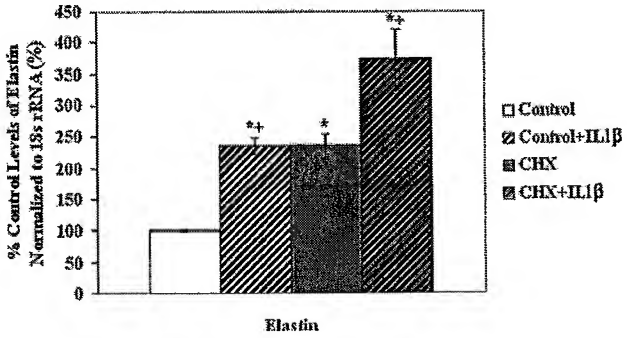

FIG. 8. The elevated steady state mRNA level of elastin induced by IL-1β was independent of ongoing protein synthesis. Cycloheximide at 10 μg/mL did not block the upregulation of elastin induced by IL-1β, even though cycloheximide alone increased the steady state level of elastin mRNA. Triplicate samples were used in each data point and the experiment was repeated 3 times. CHX: cycloheximide; IL: interleukin.

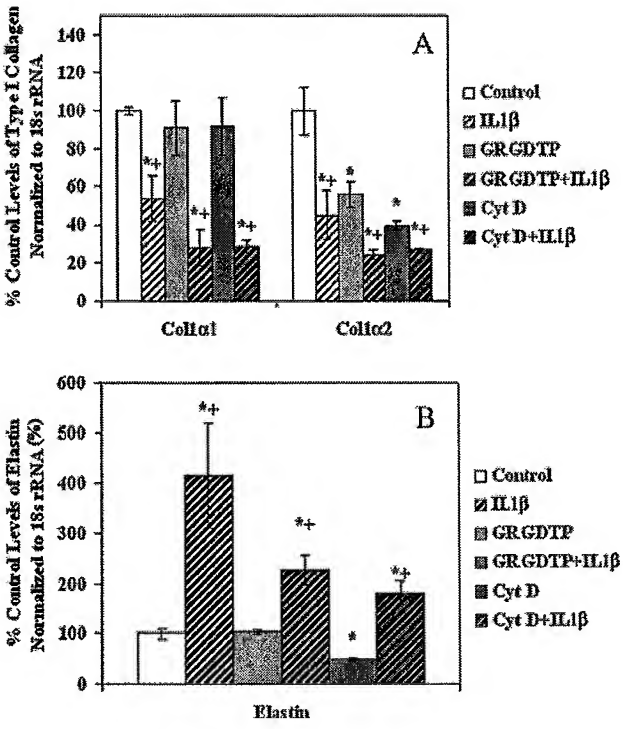

FIG. 9. Effects of IL-1β on the expression of type I collagen and elastin in the absence and presence of the peptide GRGDTP or cytochalasin D. Three BATs from each group were collected on day 5 post-addition of 100 pM of IL-1β. **(A)** Cytochalasin D at 10 μM reduced the basal level of the steady-state mRNA of elastin ($p < 0.001$). **(B)** Basal levels of the steady-state mRNA of type I collagen α_2 chain was reduced by both 100 μg/mL peptide GRGDTP and cytochalasin D, whereas α_1 chain expression was not changed by either. Neither GRGDTP peptide nor cytochalasin D at the experimental conditions blocked the effects of IL-1β on the expression of type I collagen or elastin. The experiment was repeated 3 times. Cyt: cytochalasin D; IL: interleukin.

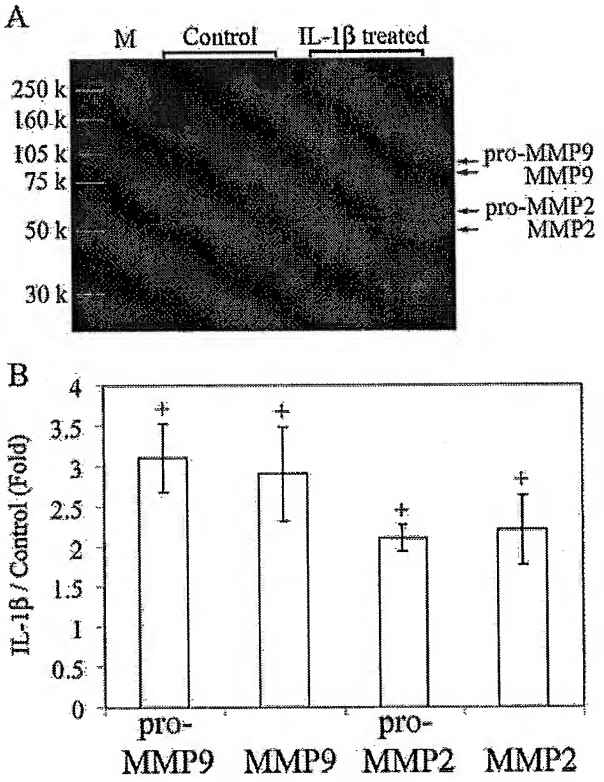

FIG. 10. Zymography of conditioned medium from control and IL-1β–treated BATs. Medium with or without IL-1β was collected on day 5 post-addition of IL-1β. The gelatinase activity in the medium was assayed using gelatin zymography as described in *Methods*. Pro–MMP-9 and MMP-9 were increased by approximately 3-fold, whereas pro–MMP-2 and MMP-2 were increased by around 2-fold ($p < 0.001$). Triplicate samples from each group were used and the experiment was repeated 3 times. Lane M was full-range rainbow molecular weight marker (units are in Daltons) from Amersham Biosciences (Piscataway, NJ). IL: interleukin; MMP: matrix metalloproteinases.

steady-state mRNA level of elastin in the presence of cycloheximide may be due to the stabilization of elastin mRNA as reported for other genes.[46]

IL-1β upregulated the expression of type I collagen in low prostaglandin E_2 (PGE_2)-producing cells, but downregulated the expression of type I collagen in higher PGE_2-producing cells.[33] We reported that IL-1β stimulated the expression of cyclooxygenase 2 (COX2) in human tenocytes,[34] which may increase the production of PGE_2 in human tenocytes and result in the reduction of type I collagen.[47]

Elastin expression responded to IL-1β at a lower dose and earlier time point compared to that of type I collagen. Therefore, it is possible to selectively upregulate the expression of elastin while maintaining type I collagen expression. The result is that one has more latitude in modulating matrix stiffness by IL-1β.

A recent study showed that cytochalasin D decreased the expression of type I collagen α_1 chain in rat tail tendon cells grown in 3D collagen gels.[48] In the present study, the expression of type I collagen α_2 chains was downregulated by cytochalasin D in human tenocytes-populated BATs. Together, these results suggest that downregulation of type I collagen by cytochalasin D may be a general phenomenon. Similar results were observed for the peptide GRGDTP-treated BATs. It is well known that disruption of the cytoskeleton or blocking cell attachment dramatically reduces cell intrinsic strain.[49–52] The balance between cell intrinsic strain and external matrix strain may be important for maintaining the integrity of tendon tissues.[53–56] This idea was also supported by the results from previous and the present studies on IL-1β.[57] IL-1β reduced both the cell intrinsic strain and extracellular matrix stiffness. The mechanism by which this phenomenon may act likely involves resetting the "setpoint" for "intrinsic strain" in the cell.[49,52,58–60] Regulation of a cell's strain setpoint includes integrin and other contacts with the substrate, cell–cell

contacts, and structural "tensegrity" components within the cell, including the cytoskeleton.[52] Modulating single or multiple components of this mechanosensory complex may permit an optimization of the matrix material properties as well as the phenotype of the cell during the anabolic phase of tissue growth *in vitro*. This concept is an extension of the general "Functional Tissue Engineering" thesis that has been proposed.[7] However, there is an additive effect of IL-1β and cytochalasin D on the expression of type I collagen, which indicates that IL-1β and cytochalasin D may activate different intracellular signal transduction pathways.

Previous studies showed that MMPs play important roles in extracellular matrix remodeling by degrading matrix proteins.[28] This is especially important for the remodeling of long-lived proteins, such as elastin.[29] We and other groups have shown that IL-1β upregulated the expression of collagenases in tendon cells.[34,35] It was reported that stress deprivation upregulated the expression of MMP-1, which contributes to the stress-deprivation–induced decrease in failure strain of tendons.[36] Overexpression of collagenases further reduces the net deposition of type I collagen in the matrix and results in the further reduction of UTS. In the present study, we showed that IL-1β also upregulated the expression of MMP-2 and -9 in human tenocytes. Despite the overexpression of gelatinases, a net increase in elastin proteins was observed in IL-1β–treated BATs. Elastin is a long-lived protein with no appreciable turnover.[29] Therefore, elevated MMP-2 and -9 may facilitate the reorganization of elastin and modulation of matrix mechanical properties.[29,61] In bone metabolism, bone resorption occurs prior to the deposition of new bone.[62] Therefore, it is predicted that an increase in MMP production may be the first step in connective tissue remodeling and an early cellular response to regulate the stiffness of the cell and its surrounding extracellular matrix.[28]

In conclusion, IL-1β increased the elasticity of BATs by differentially regulating the expression of major matrix proteins, type I collagen, and elastin, and upregulating the expression of MMPs (Fig. 11). The increased elasticity prevented BATs from rupture caused by mechanical loading. We have reported that IL-1β increased cell survival in human tenocyte-populated BATs subjected to mechanical

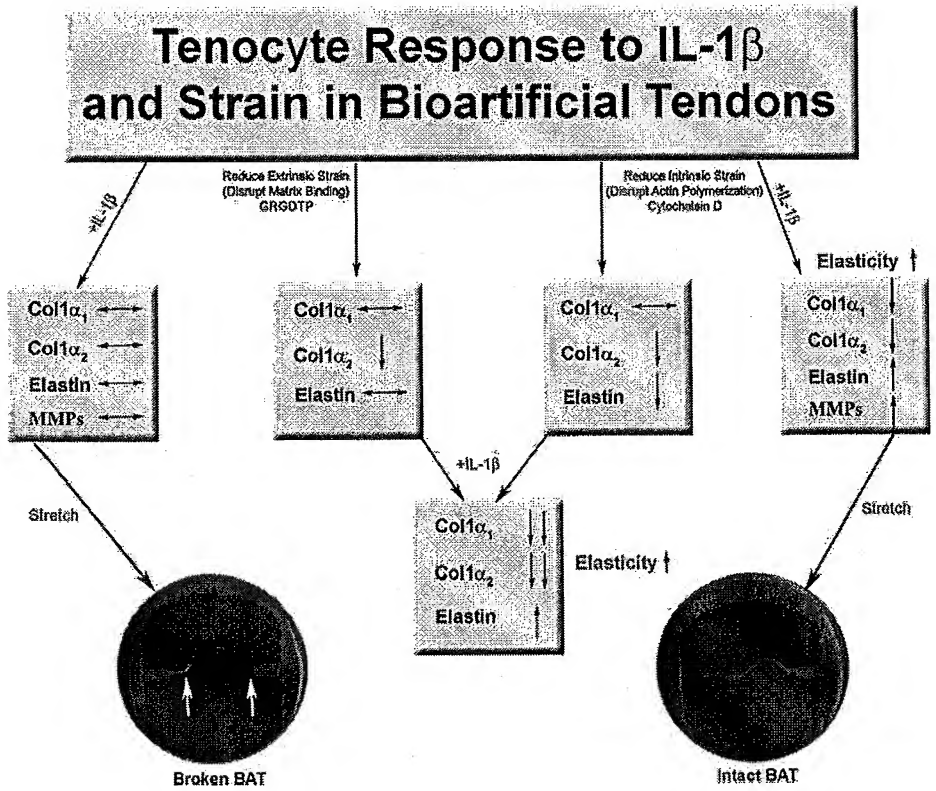

FIG. 11. Model for the regulation of type I collagen and elastin gene expression in human tenocytes by IL-1β. IL-1β treatment downregulated type I collagen expression and upregulated the expression of elastin and MMPs in human tenocytes. Reduction in intrinsic strain by cytochalasin D or the peptide, GRGDTP, downregulated the expression of elastin and/or type I collagen α_2 chain. In the presence of IL-1β, expression of both α_1 and α_2 chains were further downregulated, whereas elastin expression was upregulated. The increased elasticity prevented BATs from rupture caused by applied extrinsic strain. BAT: bioartificial tendon; Col: collagen; IL-1β: interleukin 13; MMP: matrix metalloproteinases.

loading.[57] These results suggest that IL-1β may act as a defense/survival factor in early cellular responses to normal mechanical loading or overuse. IL-1β may be used as a modulator of material properties so that engineered tissues can better match the biomechanical demands *in vivo*.

ACKNOWLEDGMENTS

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EXHIBIT C

The hypothesis of 'biophysical matrix contraction': wound contraction revisited

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ABSTRACT

Wound contraction is an orchestrated phenomenon that contributes to closure of wounds that heal by secondary intention. However, excessive and premature contraction results in scarring. Although the exact mechanism of contraction is unknown, the wound closure process is accompanied by and followed by changes in the physical and mechanical properties of the wound and periwound tissues during the biological transformation. Transforming growth factor-β (TGF-β) induces a contractile phenotype in the cellular–extracellular matrix. Meanwhile, various external and internal mechanical stresses lead to microdeformations of the wound milieu with resultant upregulation of TGF-β. Furthermore, the mechanical strain exerted on collagen fibres and other piezoelectric tissues leads to development of piezoelectric current in the wound site, which acts synergistically with TGF-β. TGF-β and mechanical strain regulate the orientation of collagen fibres parallel with the skin surface, which minimises the induction of piezoelectricity through the action of internal forces because of improper angulation of collagen fibres and these forces. The resulting dominance of external forces guides the contractile activity towards restoration of the original unwounded tissue architecture and functional activity of the previously wounded milieu. The aforementioned contractile activity proceeds into the remodelling phase of wound healing as the level of TGF-β is reduced and myofibroblasts undergo apoptosis.

Key words: Mechanical strain • Piezoelectricity • Transforming growth factor-β • Wound contraction

INTRODUCTION

Restoration of the anatomical integrity of full-thickness excisional cutaneous wounds in adult mammals is a highly orchestrated phenomenon demanding harmonised interactions of various cellular and extracellular elements. Wound contraction, the centripetal movement of the wound periphery, plays a key role in the efficient closure of dermal wounds. However, aberrant or excessive contraction patterns are both undesirable because of impaired healing response and cosmetic and functional concerns. Wound contraction accounts for approximately a 20–30% reduction in wound size in humans and 80–90% in animals with mobile skin like rats (1,2). Several mechanisms have been proposed for the explanation of this phenomenon, each with potential strengths and drawbacks.

The most widely accepted hypothesis proposes that the cellular elements of granulation tissue – fibroblasts and myofibroblasts – generate contractile forces within the wound milieu, leading to direct inward movement of wound edges by means of cellular and extracellular connections (3–6). As an alternative, some authors believe that fibroblasts act as single units producing cell locomotion forces, leading to reorganisation of fibrous collagen lattices, which in turn causes an indirect reduction in size of the wounded area by facilitating the transmission of granulation tissue forces (7). However, the fact that blocking of collagen production has been shown to have no effect on the rate of closure contradicts the aforesaid view (8). The purse-string mechanism attributes wound contraction

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Key Points

- restoration of the anatomical integrity of full thickness excisional cutaneous wounds in adult mammals is a highly orchestrated phenomenon demanding harmonised interactions of various cellular and extracellular elements
- wound contraction, the centripetal movement of the wound periphery, plays a key role in the efficient closure of dermal wounds: however, aberrant or excessive contraction patterns are both undesirable because of impaired healing response and cosmetic and functional concerns

Key Points

- as wound healing progresses through overlapping phases, the quality and also the quantity of various constituent elements of wound milieu are modified
- the wound site is subjected to various mechanical forces that can be categorised either as extrinsic or as intrinsic based on their origin

to some structural or functional entity operating circumferentially analogous to a muscular sphincter (9). Gross *et al.* (10) showed that interruption of wound continuity through circumferential resection of a thin strip of the wound edge does not alter the rate of closure, which poses a serious argument against the purse-string mechanism. The most recent studies suggest that polarised coordinated migration of a rim of densely packed proliferative fibroblasts underlying wound edges, or so-called 'picture frame', is responsible for the centripetal movement of the wound periphery, reducing the wound surface area (10,11). Yet, the picture-frame theory cannot explain the extant difference between its own concept and the in vitro experiments underscoring the key role of central granulation tissue in the process of contraction.

THE HYPOTHESIS OF DYNAMIC BIOPHYSICAL MATRIX CONTRACTION

The wound milieu is a composite structure comprising various microenvironments with different bioanatomical and mechanical properties (12). For example, collagen fibres in vascular wall and in extracellular matrix – which are two different microenvironments – when affected by equivalent external stress vectors are strained differently and exhibit dissimilar biological responses. Evidence from finite element analysis of microdeformations of wound tissue during vacuum-assisted closure therapy confirms this notion (13). The assembly of vital/cellular and non vital/extracellular microenvironments gives rise to organised and functional macroenvironments or matrices, for example granulation tissue, which is composed of several vital and non vital elements. These functional matrices are dynamic because their structure and composition are altered over time being replaced with different functional macroenvironments. A paradigmatic example would be the substitution of granulation tissue in a dermal burn wound, partly with original cutaneous tissue and partly with scar tissue.

As wound healing progresses through overlapping phases, the quality and also the quantity of various constituent elements of wound milieu are modified (14,15). For example, during angiogenesis, the number of newly formed blood vessels increases and subsequently the structural maturation of the vessels takes place. This time-dependent wound dynamism leads to the simultaneous alteration of physical properties of the wound tissues, with ongoing biological transformation of micro- and macroenvironments, for example maturation of collagen fibres and vasculature, respectively (biophysical coupling). Biophysical coupling is the reciprocal interactions of physical and biological properties of wound milieu throughout the healing period. For instance, physical modulation of the biological characteristics of wound has been suggested (16). Moreover, physical properties of wound are modified parallel with ongoing biological transformation (14). Consequently, the distribution of mechanical stresses throughout the wound would show a dynamic trend (mechanophysical coupling). In contrast, the structural elements of the microenvironments – for instance endothelial cells or collagen fibres of blood vessels – which are the sensory units of the produced strain, would show dissimilar biological responses to the exerted mechanical stimuli in various time points throughout the healing period (biomechanical coupling). Biomechanical coupling has two major causes. First, generated strains are a major determinant of dynamic mechanical properties of living tissues. For instance, while high strain rates decrease the stiffness of skin, lower rates enhance it (17). Also, the strain detection by the sensory units, for example fibroblasts, myofibroblasts, which determines their biological response, varies over time owing to altered mechanical attributes. The biological–physical–mechanical axis interactions delineate a complicated scenario through which the changes in one element of the continuum parallel and mutually affect the others in healing wounds. Of particular importance and relevance to the present hypothesis is the dissection of the aforementioned wound-associated mechanical stimuli.

The wound site is subjected to various mechanical forces that can be categorised either as extrinsic or as intrinsic based on their origin (18). Extrinsic forces are generated in the deep and superficial periwound tissues and are transmitted through connecting elements, such as extracellular fibres running between wounded and normal tissue, to the wound site. These forces vary considerably with reference to their quality and quantity. For example, the magnitude of these forces exhibits a broad range, and variations in their natural tensile, compressive or shear direction; frequency; duration and other characteristics add to their relative non uniformity. The intrinsic forces,

which have their origin within the wound milieu (the immediate periwound tissues), either are passive like the interstitial fluid pressure (19,20) and pressure from the percolation of the inflammatory exudates to the extra-cellular matrix (ECM) (21) or exhibit an active nature like the intrinsic contractile stress caused by cell–matrix interactions (22,23). Intrinsic forces exhibit more uniformity in quality and quantity compared with extrinsic forces. Moreover, in contrast to extrinsic forces, intrinsic ones correlate with specific temporal and spatial patterns as a reflection of biological–physical–mechanical axis events. Biological–physical–mechanical axis reflects the aforementioned interactions of biological, physical and mechanical domains in healing wounds. Myofibroblasts are the main contractile units within wound tissues. Around the seventh day after wounding, the differentiation of these cells begins (24). This coincides with the upregulation of transforming growth factor (TGF)-β1. This growth factor in the presence of fibronectin (FNX) stimulates the differentiation of the myofibroblasts from precursor cells. The presence of FNX is required for mechanical loading – which is the development of isometric tension – of the myofibroblasts, unloading – release of mechanical strain – of which would result in the consequent apoptosis. The mean contractile force produced by myofibroblasts and fibroblasts, when cultured on a substratum with low elastomer stiffness, approximated 2·2 and 2·0 μN/cell, respectively (25). What is more, the forces produced by fibroblasts were unaffected by augmentation of elastomer stiffness, but forces measured for myofibroblasts increased to a mean value of 4·1 μN/cell (25). The implication is that myofibroblasts are the main mechanoresponsive cells that possess a high degree of biophysical plasticity in the wound milieu.

The exertion of mechanical stress to the healing wound produces microdeformations in the wound space. These deformations are transferred to the extant cells. It has been shown that mechanically loaded living cells can proliferate in the presence of soluble growth factors, whereas unloading leads to cell cycle arrest and eventual apoptosis. Also, microdeformations of the strained collagen fibres increase the negative electrical charge in situ (22). It has been shown that the enhanced negative charge stimulates proliferative activity of soft tissue cells in the vicinity of the mechanically loaded area (22). In this environment, the mechanical stress is distributed throughout the wound milieu and is transferred to the different elements of the extant microenvironments. Some of these elements, for example collagen fibres and DNA, possess piezoelectric properties. The contraction of these elements would result in the production of piezoelectricity. However, as mentioned previously, the amount of force exerted to these elements and thus the quantity of the piezoelectricity would be proportional to the mechanical properties of the aforesaid micro- and macroenvironments. Regarding the concept of wound dynamics, we predict that the quality and quantity of piezoelectric current may change concurrently with the tissue changes as the healing processes progress. Piezoelectricity has an important role in the healing of live tissues (23). The strain-induced electrical current modulates biological events at the cellular and the molecular level (23). It is now evident that TGF-β and electric current act synergistically to enhance the effect of each other (26,27). Electrical current has also been suggested to regulate the signalling pathway of TGF-β (28). Falanga *et al.* (29) have shown that electrical stimulation (ES) upregulates receptors for TGF-β on human dermal fibroblasts in culture. Microdeformations of the wound milieu and the resultant shear strain upregulate the expression of TGF-β (30) and also produce piezoelectricity. Hence, mechanical strain may regulate wound contraction and also extracellular matrix remodelling through modulation of the biological–physical–mechanical axis and the synergistic interaction of TGF-β and piezoelectricity. The previously mentioned web of events may provoke a biomechanical cycle whereby mechanical stimulation regulates production of piezoelectricity, which in turn modulates expression and signalling of TGF-β. Subsequently, this growth factor affects the production of piezoelectricity through alteration of the extracellular matrix, especially the mechanical properties of collagen fibres.

The biological effects of these exogenous mechanical stimuli necessitate a modulation of previous wound treatment modalities. As exogenous and endogenous mechanical stimulation enhance tissue repair, we encourage practitioners to avoid immobilisation of wound and periwound tissues during the proliferative and

Key Points

- intrinsic forces exhibit more uniformity in quality and quantity compared with extrinsic forces
- myofibroblasts are the main mechanoresponsive cells that possess a high degree of biophysical plasticity in the wound milieu
- it has been shown that mechanically loaded living cells can proliferate in the presence of soluble growth factors, whereas unloading leads to cell cycle arrest and eventual apoptosis
- it has been shown that the enhanced negative charge stimulates proliferative activity of soft tissue cells in the vicinity of the mechanically loaded area
- mechanical strain may regulate wound contraction and also extracellular matrix remodelling through modulation ofthe biological–physical–mechanical axis and the synergistic interaction of TGF-b and piezoelectricity
- the biological effects of these exogenous mechanical stimuli necessitate a modulation of previous wound treatment modalities
- as exogenous and endogenous mechanical stimulation enhance tissue repair, we encourage practitioners to avoid immobilisation of wound and periwound tissues during the proliferative and remodelling phases of healing

Key Points

- the existence of an intrinsic natural control mechanism in the target or the wounded area, to control the level of exerted mechanical stimuli, seems plausible

remodelling phases of healing. Moreover, the existence of an intrinsic natural control mechanism in the target or the wounded area, to control the level of exerted mechanical stimuli, seems plausible. A second important question is what is the mechanism through which similarity of structural and functional properties of repaired tissue is restored to its original state? Considering temporal correlation, a single mechanism must fulfil both demands.

It has been shown that a constant pretension approximating 1 MP exists in normal skin, which arises from interactions of cells with their extracellular matrix as well as from the tension, which has been incorporated into the collagen fibril network during development (18). This pretension increases the coefficient of elasticity of the skin and therefore decreases its mobility. Thus, a specific stress would produce lower strain in the wound milieu, limiting the biological effects such as generation of hydraulic signals and induction of piezoelectricity. It seems that after wounding, release of pretension at the wound site may exaggerate the impact of external mechanical stimuli. Hence, we predict that coincident with the healing process, a 'biphasic force shift'; during primary phase, both extrinsic and intrinsic forces exert their biological effect. However, during the second phase, extrinsic forces mask the effect of intrinsic forces. The mechanism for development of biphasic force has been elucidated below.

Initially, after deposition of collagen fibres, both endogenous and exogenous mechanical stresses affect the wound macroenvironment. It has been shown that an angle of 45° between applied forces and collagen fibres is necessary for maximal induction of piezoelectricity (31,32). Dynamic mechanical properties of the wound site are another regulatory mechanism. The anisotropy of collagen and other piezoelectric generating tissue elements leads to proper angulation of various force vectors within the wound tissues. However, as wound healing progresses, the orientation of collagen fibres takes a more uniform pattern. It has been suggested that the application of TGF-β reduces the anisotropy of collagen fibre orientation by 29% after 14 days in healing cutaneous wounds compared with normal unwounded skin (33). Thus, the blocking of anisotropy enhances the directional non uniformity of collagen fibres by 16·2% compared with TGF-β-treated wounds, which are 12·8% more isotropic than unwounded skin. The uniform arrangement of collagen fibres throughout the healing period call forth a second phase. In this new arrangement, collagen fibres are parallel with the skin surface. This happens because collagen fibres became rapidly oriented in the direction of the force exerted on them (34). Thus, the majority of multiplanar contractile (intrinsic) forces are perpendicular to cutaneous wounds or parallel with the skin surface. This angulation of force vectors and collagen fibres – which are the major piezoelectric element of skin – decreases the amount of intrinsic-strain-related piezoelectricity. Moreover, the increasing stiffness of granulation tissue and constant nature of intrinsic forces attenuate the production of piezoelectricity by these forces. Additionally, other molecules, for example DNA, contribute to generation of low levels of intrinsic-stress-induced piezoelectricity. At the same time, several parameters contribute to the dominancy of the extrinsic forces including cyclical and intermittent nature, higher intensity and axial variability (multiaxial nature, Figure 1) of extrinsic force vectors. Multiaxial property of extrinsic forces is the result of variation in source – adjacent tissues – and direction. Therefore, during the second phase of 'biphasic force shift', extrinsic stresses are dominant and exert

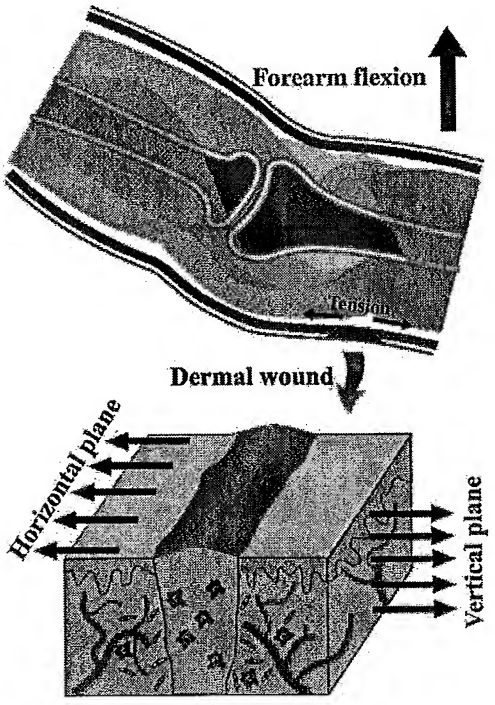

Figure 1. Development of extrinsic force vectors and multiaxial nature of these forces.

their biological effects by masking the attenuated intrinsic forces. The extrinsic (exogenous) mechanical stresses are proportional to the tissue function, anatomy and structure. So we predict that bioanatomical properties of the wound milieu and adjacent tissues determine the characteristics of piezoelectric potentials, which in turn modulate healing of wounded tissue, and this cycle proceeds through the remodelling phase. Consistent with the latter, Burgess *et al.* (35) found that mechanical stresses at the wound site may play a role in guiding collagen fibrillogenesis because altered tensions during wound closure affect the extent of scarring. Furthermore, Reger *et al.* (36) found that electrical stimulation may orient new collagen formation in a pattern similar to normal skin even in the absence of neural influences. The additional confirmative evidence comes from study of Osaki (37) who showed that the degree of orientation of collagen fibres in calf skin was greater in areas where skin motions were marked. Gradually, the cycle is coalesced with normal ongoing remodelling of repaired tissue similar to that which occurs in unwounded tissues. Through this mechanism (i) wound contraction is controlled, (ii) original tissue structure is restored and (iii) the influence of unnecessary interfering mechanical forces (peripheral mechanical fog) is minimised. Mechanical fog describes the unfavourable extrinsic mechanical stimuli developed as a result of mobility of adjacent tissues with adverse effects on the healing procedure because of their direction or magnitude. Irion *et al.* (38) have recently reported that mechanically stimulated 4-mm biopsy wounds in rats reduced time to closure by nearly 50% compared with sham-stimulated wounds.

The proposed hypothesis has important clinical implications, especially in conditions in which wound contraction is considered a serious challenge, for example plastic and reconstructive surgery and the conservative management of chronic wounds. The use of electrical stimulation for the enhancement of healing should be customised for application to various regions of human body according to mechanical properties, for example functional mobility and anatomical features such as surface curvature. Furthermore, alteration of biological–physical–mechanical axis modules through therapeutic approaches may influence the quality and quantity of wound contraction and resultant scarring.

We have described the phenomenon of tensgrity in which the cell itself behaves as a mechanical transducer, which allows mechanical events that distort the cell to be detected and routed to intracellular signalling mechanisms that produce rapid adaptation to the mechanical stress (39–41).

The future research may be directed towards evaluation of present hypothesis by in vitro and in vivo models. The investigation of effect of piezoelectricity on wound contraction through implantation of piezoelectric sensors in wounded regions seems interesting. Finally, reverse piezoelectric phenomenon as a therapeutic modality to diminish contraction and scarring may prove useful.

Key Points

- we predict that bioanatomical properties of the wound milieu and adjacent tissues determine the characteristics of piezoelectric potentials, which in turn modulate healing of wounded tissue, and this cycle proceeds through the remodelling phase
- future research may be directed towards evaluation of present hypothesis by in vitro and *in vivo* models
- reverse piezoelectric phenomenon as a therapeutic modality to diminish contraction and scarring may prove useful

GLOSSARY

Piezoelectric effect: The generation of electricity or electric polarity in dielectric crystals subjected to mechanical stress, or the generation of stress in such crystals subjected to an applied voltage.

Angulation of collagen fibres: The geometric spatial arrangement of collagen fibres that leads to formation of an angle between these fibres.

Microdeformations: Microscopic deformations of tissue.

Tensgrity: Tensgrity describes a structural-relationship principle in which structural shape is guaranteed by the finitely closed, comprehensively continuous, tensional behaviours of the system and not by the discontinuous and exclusively local compressional member behaviours. Tensgrity provides the ability to yield increasingly without ultimately breaking or coming asunder.

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